

WO 03/072014

PCT/US02/16877

Recipient (P678-54) and donor (G43:BW6169) strains were grown overnight in 10 mL of LB media (10 g NaCl, 10 g select peptone 140, and 5 g yeast extract in one liter ddH<sub>2</sub>O). The samples were centrifuged and then concentrated in about 0.2 mL of LB media. The concentrated samples were combined and incubated with slow rotation for 30 minutes at 30°C, and were then plated on LB agar plates that contained streptomycin (50 µg/mL) and tetracycline (50 µg/mL). (Ampicillin, streptomycin, tetracycline, and all other chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated.) Recipient cells were resistant to streptomycin and donor cells were resistant to tetracycline; only conjugates, which contained both resistance genes, were able to grow on the LB agar plates that contained streptomycin (50 µg/mL) and tetracycline (50 µg/mL).

Putative conjugates were screened for Lambda phage sensitivity using a cross streak technique, in which putative colonies were cross-streaked on an LB agarose plate (streptomycin, 50 µg/mL, and tetracycline, 50 µg/mL) that had been streaked with live Lambda phage. The streaked conjugate colonies were streaked perpendicular to the Lambda phage streak; if a conjugate was sensitive to Lambda phage infection then, upon contact with the Lambda phage streak, there was cell lysis and thus less or no bacterial growth. Thus, in the case of conjugates that were sensitive to Lambda phage, there was decreased bacterial growth "downstreak" from the phage streak.

The conjugate E. coli that were found to be sensitive to Lambda phage infection were then used to create Lambda lysogens. Lysogenization is a process during which Lambda phage incorporates its genome, including exogenous genes added thereto, into a specific site on the chromosome of its E. coli host cell.

The DE3 gene, which is present in the genome of the Lambda phage used to create lysogens, encodes RNA polymerase from bacteriophage T7. Lysogenation was carried out using the DE3-Lysogenation kit (Novagen, Madison, WI) essentially according to the manufacturer's instructions. A T7 polymerase dependent tester phage was used to confirm the presence and expression of the DE3 gene on the bacterial chromosome. The T7-dependent tester phage can only form plaques on a bacterial known in the presence of T7 polymerase. The phage uses a T7 promoter for expression of its essential genes. Therefore in a plaque-forming assay only cells which express T7 polymerase can be lysed by the tester phage and only these cells will allow for the formation of plaques. As is described in more detail herein, episomal expression elements that are used in minicells may be designed such

WO 03/072014

PCT/US02/16877

that transcription and translation of a cloned gene is driven by T7 RNA polymerase by utilizing expression sequences specific for the T7 RNA polymerase.

## **EXAMPLE 2: CLONING OF RAT EDG-1 INTO THE PCAL-C EXPRESSION VECTOR**

### **5 Materials**

Taq Polymerase, PCR Buffers, and PCR reagents were purchased from Roche Molecular Biochemicals (Indianapolis, IN). All restriction enzymes were purchased from Gibco BRL (Grand Island, NY) and Stratagene (La Jolla, CA). QIAprep mini and maxi kits, PCR purification Kits, RNeasy miniprep kits, and the One Step RT-PCR Kit were  
10 purchased from QIAGEN (Valencia, CA). The GeneClean Kit was purchased from BIO 101 (Carlsbad, CA). IPTG (isopropyl-beta-D-thiogalactopyranoside), T4 DNA Ligase, LB Media components and agarose were purchased from Gibco BRL. The pCAL-c prokaryote expression vector and competent cells were purchased from Stratagene.

The pCAL-c expression vector has a structure in which an ORF may be operably  
15 linked to a high-level (but T7 RNA polymerase dependent) promoter, sequences that bind the E. coli Lac repressor, and the strong T7 gene 10 ribosome-binding site (RBS). The LacI repressor is also encoded by an expressed from the pCAL-c vector. As long as it is bound to its recognition sequences in the pCAL-c expression element, the lac repressor blocks transcription from the T7 promoter. When an inducing agent, such as IPTG is added, the lac  
20 repressor is released from its binding sites and transcription proceeds from the T7 promoter, provided the T7 RNA polymerase is present. After induction, the cloned and expressed protein may constitute the majority of newly expressed cellular proteins due to the efficient transcription and translation processes of the system.

### **Amplification**

The first step in cloning rat Edg-1 (rEDG-1) into an expression vector was to design  
25 primers for amplification via PCR (polymerase chain reaction). PCR primers were designed using the rat Edg-1 sequence (Nakajima et al., Biophys. J. 78:319A, 2000) in such a manner that they contained either sites for NheI (GCTAGC) or BamHI (GGATCC) on their five prime ends. The upstream primer had the sequence of SEQ ID NO:31. The three prime  
30 downstream primer (SEQ ID NO:32) also contained a stop codon, as the pCAL-c vector contains a Calmodulin Binding Protein (CBP) "tag" at its carboxyl terminus which was not

WO 03/072014

PCT/US02/16877

intended to be incorporated into the rat Edg-1 polypeptide in this expression construct. The primer and resulting PCR products were designed so that the five prime end of the rat Edg-1 ORF was in frame with the methionine start codon found in the pCAL-c vector.

#### OLIGONUCLEOTIDE PRIMER SEQUENCES FOR CLONING INTO PCAL-C:

##### 5 Edg1/pCAL-c construct primers:

Upstream primer (SEQ ID NO:31)

5' - AATTGCTAGCTCCACCAGCATCCCAGTGGTTA-3'

Downstream primer (SEQ ID NO:32)

5' - AATTGGATCCTTAAGAAGAAGAATTGACGTTT-3'

##### 10 Edg1/CBP fusion construct primers:

Upstream primer (SEQ ID NO:31)

5' - AATTGCTAGCTCCACCAGCATCCCAGTGGTTA-3'

Downstream primer (SEQ ID NO:33)

5' - AATTGGATCAGAGAAGAATTGACGTTTCCA-3'

##### 15 Edg1/His6 construct primers:

Upstream primer (SEQ ID NO:31)

5' - AATTGCTAGCTCCACCAGCATCCCAGTGGTTA-3'

Downstream primer (SEQ ID NO:34)

5' -

##### 20 AATTGGATCCTTAATGATGATGATGATGATGATGAGAAGAAGAATTGACGTTTCC-3'

##### Edg3/rtPCR primers:

Upstream primer (SEQ ID NO:35)

5' - TTATGGCAACCACGCACGCGCAGG-3'

Downstream primer (SEQ ID NO:36)

##### 25 5' - AGACCGTCACTTGACAGGAC-3'

##### Edg3/pCAL-c construct primers:

Upstream primer (SEQ ID NO:37)

5' - AATTGCTAGCACGCACGCGCAGGGGCACCCGC-3'

Downstream primer (SEQ ID NO:38)

##### 30 5' - AATTGGTACCTCACTTGACAGGACCCCATTCG-3'

##### Edg3/His6 construct primers:

Upstream primer (SEQ ID NO:39)

5' - AATTGCTAGCACGCACGCGCAGGGGCACCCGC-3'

Downstream primer (SEQ ID NO:16)

WO 03/072014

PCT/US02/16877

5' -

AATTGGTACCTCAATGATGATGATGATGATGCTTGCAGAGGACCCCATCTG-3'

**GFP/pCAL-c construct primers:**

5 Upstream primer (SEQ ID NO:40)

5' -GGTCGCCACCATGGTGAGCAA-3'

Downstream primer (SEQ ID NO:41)

5' -TTAAGGATCCTTACTTGTACAGCTCGTCCAT-3'

**GFP/CBP construct primers:**

10 Upstream primer (SEQ ID NO:42)

5' -GGTCGCCACCATGGTGAGCAA-3'

Downstream primer (SEQ ID NO:43)

5' -TTAAGGATCCTTGTACAGCTCGTCCATGCC-3'

**Notes:**

15 Restriction endonuclease sites are underlined

Stop codons are double underlined

The primers were used to amplify the rEdg-1 DNA ORF using the polymerase chain reaction (PCR). The template used for amplification was mRNA isolated from rat muscle tissue using the RNeasy Miniprep Kit (Qiagen) and was carried out essentially according to the manufacturer's protocol. Both the rtPCR and PCR amplification steps were carried out in a single reaction using the One Step RT-PCR Kit (Qiagen). The resulting rat Edg-1 PCR fragment was purified using the PCR Purification Kit (Qiagen). The amplified double stranded rEdg-1 DNA sequence contained the NheI site at the 5-prime end and the BamHI site at the 3-prime end. This amplified rEdg-1 fragment was used for cloning into the pCAL-c expression vector.

The pCAL-c expression vector contains NcoI, NheI, and BamHI restriction sites in its multiple cloning site. In order to insert rEdg-1-encoding sequence into the expression vector, the rEdg-1 PCR fragment and the pCAL-c expression vector were digested with NheI and BamHI restriction enzymes for one hour at 37°C. The reaction mixture for the digestion step consisted of 1 µg of DNA, 1x restriction buffer, and 1 µL of each enzyme. The reaction mixture was brought to a final volume of 20 µL with ddH<sub>2</sub>O (dd, double distilled). After 45 minutes, 1 µL of Calf Intestine Alkaline Phosphatase (CIAP) was added to the pCAL-c reaction mixture in order to remove the terminal phosphates from the digested plasmid DNA.

WO 03/072014

PCT/US02/16877

The reactions were incubated for an additional 15 minutes at 37°C. The digested DNA samples were then run on a 1% TAE (Tris-acetate/EDTA electrophoresis buffer) agarose gel at 130 volts for 45 minutes. The bands were visualized with UV light after the gel was stained with ethidium bromide.

- 5           The appropriate bands were cut out of the gel for purification using the GeneClean Kit (BIO101). The Purified DNA fragments were then quantified on a 1% TAE agarose gel. For the ligation reaction, ratios of insert to vector of 6:1 and 3:1 were used. A negative control comprising vector only was also included in the ligation reactions. The reaction mixtures contained insert and vector DNA, 4 µL Ligase buffer, and 2 µL Ligase. The
- 10          reaction was brought up to a final volume of 20 µL with ddH<sub>2</sub>O. The ligation was carried out at room temperature for about 2 hours. Ten (10) µL of the ligation reaction mixture was used for subsequent transformation steps.

- Ligated DNA was introduced into Epicurian Coli XL1-Blue competent cells using the heat shock transformation technique as follows. The ligation mixture was added to 100 µL of
- 15          competent cells, placed on ice, and was incubated for about 30 minutes. The cells were then heat shocked at 37°C for 1 minute and put back on ice for 2 minutes. Following heat shock, 950 µL of room temperature LB media was added to the cells and the cells were shaken at 37°C for 1 hour. Following the 1-hour agitation the cells were pelleted for one minute at 12000 rpm in a Eppendorf 5417C microcentrifuge. The supernatant was carefully poured off
- 20          so that about 200 µL remained. The cells were then resuspended in the remaining LB media and spread on 100x15 mm LB agarose plates containing 50 µg/mL ampicillin. The plates were incubated overnight at 37°C. Colonies were counted the following day, and the ratio of colonies between the negative control and the ligated samples was determined. A high ratio of the number of colonies when the ligation mixture was used to transform cells, as
- 25          contrasted to the number of negative control colonies indicated that the cloning was successful. Transformed colonies were identified, isolated, and grown overnight in LB media in the presence of ampicillin. The resulting bacterial populations were screened for the presence of the Edg-1-pCAL-c expression construct.

- Plasmid DNA was isolated from the cells using the QIAprep Spin Miniprep Kit
- 30          (Qiagen). Isolated Edg-1-pCAL-c constructs were screened using the restriction enzyme ApaI, which digests the Edg-1-pCAL-c construct at two different sites: one in the Edg-1 coding sequence and one in the pCAL-c vector itself. The plasmid preparations were digested

WO 03/072014

PCT/US02/16877

with ApaI electrophoresed on a 1% TAE agarose gel and visualized using uv light and ethidium bromide staining. The predicted sizes of the expected DNA fragments were 2065 bp and 4913 bp. As shown in Figure 3, bands of the predicted size were present on the gel. The entire Edg-1-pCAL-c construct was sequenced in order to confirm its structure. This expression construct, a pCAL-c derivative that contains the rat Edg-1 ORF operably linked to a T7 promoter and lac repressor binding sites, is designated "prEDG-1" herein.

#### EXAMPLE 3: CONSTRUCTION OF RAT EDG-1-CBP FUSION PROTEIN

In order to detect rat Edg-1 protein expression, rEdg-1 coding sequences were cloned into the pCAL-c vector in frame with a CBP fusion tag. The cloning strategy for the rEdg-1-CBP construct was performed essentially as described for the Edg-1-pCAL-c construct with the following differences. The PCR primers (SEQ ID NOS:3 and 5) were as described for the Edg-1-pCAL-c cloning except for the omission of the stop codon in the downstream primer (SEQ ID NO:33). The removal of the stop codon is required for the construction of the Edg-1-CBP fusion protein. The pCAL-c vector is designed so that, when the BamHI site is used for insertional cloning, and no stop codon is present in an ORF inserted into the pCAL-c expression vector the cloned ORF will be in-frame with the CBP fusion tag. Because the three prime downstream primer did not contain a stop codon, a CBP fusion tag could be cloned in-frame with the Edg-1 ORF. Other cloning steps were performed essentially as described before. The resulting plasmid, a pCAL-c derivative that comprises an ORF encoding a rat Edg-1-CBP fusion protein operably linked to a T7 promoter and lac repressor binding sites, is designated "prEDG-1-CBP" herein.

#### EXAMPLE 4: CLONING OF A HIS-TAGGED RAT EDG-1 INTO PCAL-C EXPRESSION VECTOR

The rEdg-1 protein was manipulated to generate a fusion protein having a 6xHis tag at its carboxyl terminus. A "6xHis tag" or "His tag" is an amino acid sequence consisting of six contiguous histidine residues that can be used as an epitope for the binding of anti-6xHis antibodies, or as ligand for binding nickel atoms. The His-tagged rEdg-1 fusion protein is used to detect rEdg-1 protein expression in the minicell expression system environment.

The rEdg-1-6xHis construct was cloned using the strategy described above for the construction of the rEdg-1-pCAL-c expression construct (prEDG-1), with the upstream primer having the sequence of SEQ ID NO:3, but with the exception that the three prime

WO 03/072014

PCT/US02/16877

downstream primer (SEQ ID NO:34) was designed to contain six histidine codons followed by a stop codon. The 18 base pair 6xHis tag was incorporated into the carboxyl terminus of the Edg-1 protein as expressed from the pCAL-c vector. Subsequent cloning procedures (PCR, restriction digest, gel purification, ligation, transformation, etc.) were performed as described previously for the Edg-1-pCAL-c construct (prEDG-1). The resulting plasmid, a pCAL-c derivative that comprises an ORF encoding a carboxy-terminal His-tagged rat Edg-1-CBP fusion protein operably linked to a T7 promoter and lac repressor binding sites, is designated "prEDG-1-6xHis" herein.

**EXAMPLE 5: AMPLIFICATION AND CLONING OF RAT EDG-3 SEQUENCES**

The Edg-3 full length coding sequence was amplified via PCR from rat skeletal muscle mRNA using primers (SEQ ID NOS:35 and 36) designed from the known mouse sequence (Genbank accession NM\_010101). The mRNA used as a template for the amplification reaction was isolated using the RNeasy Miniprep Kit (Qiagen). Both the rtPCR and PCR amplification steps were carried out in a single reaction using the One Step RT-PCR Kit (Qiagen). The rEdg-3 PCR products were visualized with UV after electrophoresis in 1% TAE agarose gels and ethidium bromide staining.

The predicted size of the amplified PCR products is 1145 base pairs. An appropriately-sized DNA band was isolated from the TAE gel and purified using the GeneClean Kit (BIO101). The purified band was ligated to the pCR3.1 vector using the TA-cloning kit (Invitrogen). Other cloning steps were carried out as described previously for the cloning of the rEdg-1-pCAL-c construct (prEDG-1) with the exception that the samples were screened using the EcoRI restriction enzyme. The expected sizes of the digested bands were 1145 base pairs and 5060 base pairs. Positive clones were analyzed by automated sequencing. The nucleotide sequences were analyzed using BLAST searches from the NCBI web site ([www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)). The predicted full length rat Edg-3 amino acid sequence was assembled from the nucleotide sequencing data using in silico translation. The pCR3.1 vector comprising the rat Edg-3 ORF is designated "pCR-rEDG-3" herein.

**EXAMPLE 6: CLONING OF RAT EDG-3 CODING SEQUENCES INTO THE PCAL-C EXPRESSION VECTOR**

In order to express it in the minicell expression system, the rat Edg-3 ORF was cloned into the pCAL-c expression vector. The cloning strategy used was as described above for the cloning of the rat Edg-1 gene into the pCAL-c vector with the following exceptions.

WO 03/072014

PCT/US02/16877

The primers used for PCR amplification were designed from the rat Edg-3 sequence and contained sites for the restriction enzymes *NheI* and *KpnI* (GGTACC). The *NheI* site was added to the five prime upstream primer (SEQ ID NO:37) and the *KpnI* site was added to the three prime downstream primer; SEQ ID NO:38). The *NheI* and *KpnI* restriction enzymes were used for the digestion reaction. The reaction mixture for the digestion step consisted of 1 µg of DNA, 1x restriction buffer (provided with the enzyme), and 1 µL of each enzyme. Plasmid preparations were screened by digestion with *NheI* and *KpnI*. The digested plasmid DNA was electrophoresed on a TAE agarose gel and visualized by UV after staining with ethidium bromide. The resultant band sizes were predicted to be 1145 base pairs and 5782 base pairs. The positive plasmid clones were analyzed with automated sequencing. The resulting plasmid, a pCAL-c derivative that comprises an ORF encoding a rat Edg-3 protein operably linked to a T7 promoter and lac repressor binding sites, is designated "pEDG-3" herein.

**EXAMPLE 7: CLONING OF A HIS-TAGGED RAT EDG-3 INTO THE PCAL-C EXPRESSION VECTOR**

In order to detect expression of the rat Edg-3 protein in the minicell expression system, the rat Edg-3 coding sequence was manipulated so as to contain a 6xHis tag at the carboxyl terminus of the protein. The cloning strategy used to create this construct was essentially the same as described above for the rEdg-3-pCAL-c (prEDG-3) construct cloning, with the upstream primer having the sequence of SEQ ID NO:37, with the exception that the three-prime downstream primer (SEQ ID NO:18) was designed to contain a 6xHis coding sequence followed by a stop codon, which allowed for the incorporation of the 6xHis amino acid sequence onto the carboxyl terminus of the Edg-3 receptor protein. Other cloning and screening steps were performed as described above. The resulting plasmid, a pCAL-c derivative that comprises an ORF encoding a carboxy-terminal His-tagged rat Edg-3 fusion protein operably linked to a T7 promoter and lac repressor binding sites, is designated "prEDG-3-6xHis" herein.

**EXAMPLE 8: GFP CLONING INTO PCAL-C EXPRESSION CONSTRUCT**

Cloning of GFP-encoding nucleotide sequences into the pCAL-c vector was performed in order to produce an expression construct having a reporter gene that can be used to detect protein expression (GFP, green fluorescent protein). The cloning strategy used was essentially the same as the cloning strategy described above with the following exceptions. The template used for PCR amplification was the peGFP plasmid "construct"

(GFP construct sold by Clontech). The primers used for amplification were designed from the GFP coding sequence and contained sites for the restriction enzymes NcoI and BamHI. The NcoI site was added to the five prime upstream primer (SEQ ID NO:40) and the BamHI site was added to the three prime downstream primer; see SEQ ID NO:41) The NcoI and BamHI restriction enzymes were used for the digestion reaction. The reaction mixture for the digestion step consisted of 1 µg of DNA, 1x restriction buffer (provided with the enzyme), and 1 µL of each enzyme. The screening of the plasmid preparations was carried out using NcoI and BamHI. Digested plasmid preparations were electrophoresed and visualized on TAE agarose gels with UV after staining with ethidium bromide. Restriction products having the predicted sizes of 797 and 5782 base pairs were seen. Positive plasmid clones were sequenced using an automated sequencer. The resulting plasmid, a pCAL-c derivative that comprises an ORF encoding a rEdg-3-GFP fusion protein operably linked to a T7 promoter and lac repressor binding sites, is designated "prEDG-3-GFP" herein.

**EXAMPLE 9: DESIGN CONSTRUCTION OF CONTROL EXPRESSION ELEMENTS**

Control expression elements used to detect and quantify expression of proteins in minicells were preposed. These controls direct the expression of detectable proteins. An expression element used as positive control is pPTC12, which is supplied with the pCAL-c expression vector from Stratagene. This construct contains an ORF encoding a fusion protein comprising beta-galactosidase linked to CBP. Induction of expression of pTC12 should result in the production of a protein of about 120 kD, and this protein is detected via its enzymatic activity or by using antibodies directed to epitopes on the beta-galactosidase or CBP polypeptide.

A GFP fusion construct was created and used as a positive control for the CBP detection kit. This construct was a positive control for induction of protein expression in the minicell expression system. The cloning strategy used to create the construct was essentially the same as that used for the cloning of the GFP into the pCAL-c expression vector, with the exception that the three prime downstream primer did not contain a stop codon; this allowed for the in frame incorporation of the CBP fusion tag to the GFP protein. The upstream primer had the sequence of SEQ ID NO:42, and the downstream primer had the sequence SEQ ID NO:43. The nucleotide sequence of the expression element was confirmed using an automated sequencer. The resulting plasmid, a pCAL-c derivative that comprises an ORF

WO 03/072014

PCT/US02/16877

encoding GFP operably linked to a T7 promoter and lac repressor binding sites, is designated "pGFP-CBP" herein.

**EXAMPLE 10: INTRODUCTION OF PCAL-C EXPRESSION CONSTRUCTS INTO THE MC-T7 ESCHERICHIA COLI STRAIN**

5 The MC-T7 *E. coli* strain was made competent using the  $\text{CaCl}_2$  technique. In brief, cells were grown in 40 mL LB medium to an  $\text{OD}_{600}$  of 0.6 to 0.8, and then centrifuged at 8000 rpm (7,700 g) for 5 min at 4°C. The pellet was resuspended in 20 mL of cold  $\text{CaCl}_2$  and left on ice for five minutes. The cells were then centrifuged at 8000 rpm (7,700 g) for 5 min at 4°C. The cell pellet was resuspended in 1 mL of cold  $\text{CaCl}_2$  and incubated on ice for 10 30 min. Following this incubation 1 mL of 25% glycerol was added to the cells and they were distributed and frozen in 200  $\mu\text{L}$  aliquots. Liquid nitrogen was used to freeze the cells. These cells subsequently then used for the transformation of expression constructs.

**EXAMPLE 11: PREPARATION OF MINICELLS**

To some degree, the preparation of minicells varied according to the type of 15 expression approach that is used. In general, there are two such approaches, although it should be noted from the outset that these approaches are neither limiting nor mutually exclusive. One approach is designed to isolate minicells that already contain an expressed therapeutic protein or nucleic acid. Another approach is designed to isolate minicells that will express the protein or nucleic acid in the minicell following isolation.

20 *E. coli* are inoculated into bacterial growth media (e.g., Luria broth) and grown overnight. After this, the overall protocol varies with regards to methods of induction of expression. The minicell producing cultures used to express protein post isolation are diluted and grown to the desired  $\text{OD}_{600}$  or  $\text{OD}_{450}$ , typically in the log growth phase of bacterial cultures. The cultures are then induced with IPTG and then isolated. The IPTG 25 concentration and exposure depended on which construct was being used, but was usually about 500  $\mu\text{M}$  final for a short time, typically about 4 hours. This treatment results in the production of the T7 polymerase, which is under control of the LacUVR5 promoter, which is repressed by the LacI repressor protein. IPTG relieves the LacI repression and thus induces expression from the LacUVR5 promoter which controls expression of the T7 polymerase 30 from the chromosome. This promoter is "leaky" that is, there is always a basal level of T7 polymerase which can be selected for or against so that the induction before isolation is not required. (This induction step is not required if a non-T7 expression system is used, as the

The *E. coli* cultures that produce minicells containing a therapeutic protein or nucleic acid have different induction protocols. The overnight cultures are diluted as described above; however, in the case of proteins that are not toxic to the parent cells, this time the media used for dilution already contains IPTG. The cultures are then grown to mid-log growth and minicells are isolated. These cultures produce the therapeutic protein or nucleic acid as they grow, and the minicells derived therefrom contain the therapeutic protein or nucleic acid.

Alternatively or additionally, IPTG is added and expression is induced after the isolation of minicells. In the case of non-toxic proteins or nucleic acids that are expressed from expression elements in minicells, this treatment enhances production of the episomally encoded gene product. In the case of toxic gene products induction post-isolation is preferred.

### EXAMPLE 12: MINICELL ISOLATION

Minicells were isolated from the minicell producing MC-T7 strain of *E. coli* using centrifugation techniques. The protocol that was used is essentially that of Jamnati pour et al. (Translocation of *Vibrio Harveyi* N<sub>2</sub>N'-Diacetylchitobiase to the Outer Membrane of *Escherichia Coli*, *J. Bacteriol.* 169: 3785-3791, 1987) and Matsumura et al. (Synthesis of Mot and Che Products of *Escherichia coli* Programmed by Hybrid ColE1 Plasmids in Minicells, *J. Bacteriol.* 132:996-1002, 1977).

In brief, MC-T7 cells were grown overnight at 37°C in 2 to 3 mL of LB media containing ampicillin (50 µg/mL), streptomycin (50 µg/mL), and tetracycline (50 µg/mL). (ampicillin was used only when growing MC-T7 cells containing a pCAL-c expression construct). The cells were diluted 1:100 in a total volume of 100 to 200 mL LB media with antibiotics, and grown at 37°C until they reached an OD<sub>600</sub> of 0.4 to 0.6, which is roughly beginning of the log growth phase for the MC-T7 *E. coli*. During this incubation the remainder of the overnight culture was screened for the presence of the correct expression construct using the techniques described above. When the cultures reached the appropriate OD<sub>600</sub> they were transferred to 250 mL GS3 centrifuge bottles and centrifuged (Beckman

WO 03/072014

PCT/US02/16877

centrifuge) at 4500 rpm (3,500 g) for 5 min. At this point the supernatant contains mostly minicells, although a few relatively small whole cells may be present.

The supernatant was transferred to a clean 250 mL GS3 centrifuge bottle and centrifuged at 8000 rpm (11,300 g) for 10 min. The pellet was resuspended in 2 mL of 1x BSG (10x BSG: 85 g NaCl, 3 g  $\text{KH}_2\text{PO}_4$ , 6 g  $\text{Na}_2\text{HPO}_4$ , and 1 g gelatin in 1 L ddH<sub>2</sub>O) and layered onto a 32 mL 5 to 20% continuous sucrose gradient. The sucrose gradient was made with sucrose dissolved in 1x BSG.

The sucrose gradient was then loaded in a Beckman SW24 rotor and centrifuged in a Beckman Ultracentrifuge at 4500 rpm (9,000 g) for 14 min. Following ultracentrifugation a single diffuse band of minicells was present. The top two thirds of this band was aspirated using a 10 mL pipette and transferred to a 30 mL Oakridge tube containing 10 mL of 1x BSG. The sample was then centrifuged at 13,000 rpm (20,400 g) for 8 min. Following centrifugation, the pellet was resuspended in 2 mL 1x BSG, and the resuspended cells were loaded onto another 5 to 20% sucrose gradient. This sucrose gradient was centrifuged and the minicells were collected as described above. The sucrose gradient procedure was repeated a total of three times.

Following the final sucrose gradient step the entire minicell band was collected from the sucrose gradient and added to a 30 mL Oakridge tube that contained 10 mL of MMM buffer (200 mL 1x M9 salts, 2 mL 20% glucose, and 2.4 mL DIFCO Methionine Assay Medium). This minicell solution was centrifuged at 13,000 rpm (20,400 g) for 8 min. The pellet was resuspended in 1 mL of MMM Buffer.

The concentration of minicells was determined using a spectrophotometer. The OD<sub>450</sub> was obtained by reading a sample of minicells that was diluted 1:100.

### EXAMPLE 13: OTHER METHODS TO PREPARE AND ISOLATE MINICELLS

By way of non-limiting example, induction of *E. coli* parental cells to form minicells may occur by overexpression of the *E. coli* *ftsZ* gene. To accomplish this both plasmid-based and chromosomal overexpression constructs were created that place the *ftsZ* gene under the control of various regulatory elements (Table 6).

WO 03/072014

PCT/US02/16877

**TABLE 6. REGULATORY CONSTRUCTS CONTROLLING FTSZ EXPRESSION.**

Regulatory region	inducer	[inducer]	SEQ ID NO.:
Para::ftsZ	Arabinose	10 mM	1, 3
Prha::ftsZ	Rhamnose	1 mM	2, 4
Ptac::ftsZ	IPTG	30 $\mu$ M	5, Garrido et al. <sup>a</sup>

a. Garrido, T. et al. 1993. Transcription of *ftsZ* oscillates during the cell cycle of *Escherichia coli*.

5 Oligonucleotide names and PCR reactions use the following format:

- “gene-1” is N-terminal, 100% homology oligo for chromosomal or cDNA amplification
- “gene-2” is C-terminal, 100% homology oligo for chromosomal or cDNA amplification
- “gene-1-RE site” is same sequence as gene-1 with additional residues for remainder of sequence, RE sites, and/or chimeric fusions.
- “gene-2-RE site” is same sequence as gene-1 with additional residues for remainder of sequence, RE sites, and/or chimeric fusions.

Use “gene-1, 2” combo for chromosomal/cDNA amplification and “gene-1 RE site, gene-2-RE site” to amplify the mature sequence from the “gene-1, 2” gel-purified product.

**TABLE 7: OLIGONUCLEOTIDE PRIMER SEQUENCES FOR TABLE 6 CONSTRUCTS**

SEQ ID NO.:	Primer name	5' to 3' sequence
44	FtsZ-1	CCAATGGAACCTACCAATGACGCGG
45	FtsZ-2	GCTTGCTTACGCAGGAATGCTGGG
46	FtsZ-1-PstI	CGCGGCTGCAGATGTTTGAACCAATGGAACCTACCAA TGACGCGG
47	FtsZ-2-XbaI	GCGCCTCTAGATTATTAATCAGCTTGCTTACGCAGGAA TGCTGGG

Table 7 oligonucleotide sequences are for use in cloning *ftsZ* into SEQ ID NO.:1 and 2 (insertions of *ftsZ* behind the arabinose promotor (SEQ ID NO.: 1) and the rhamnose promotor (SEQ ID NO.: 2).

**TABLE 8: OLIGONUCLEOTIDE PRIMER SEQUENCES FOR FTSZ CHROMOSOMAL DUPLICATION CONSTRUCTS**

SEQ ID NO.:	Primer name	5' to 3' sequence
48	Kan-1	GCTAGACTGGGCGGTTTTATGGACAGCAAGC
49	Kan-2	GCGTTAATAATTGAGAAGAACTCGTCAAGAAGGCG
50	Kan-1-X-frt	GCGCCTACTGACGTAGTTCGACCGTCTGGACTAGCGAAG TTCCTATACTTTCTAGAGAATAGGAACCTTCGCTAGACTG GGCGGTTTTATGGACAGCAAGC
51	Kan-2-intD-frt	CAAGATGCTTTGCCTTTGTCTGAGTTGATACTGGCTTTG GGAAAGTTCCTATTCTCTAGAAAGTATAGGAACCTTCGCGT TAATAATTGAGAAGAACTCGTCAAGAAGGCG
52	AraC-1	CGTTACCAATTATGACAACCTTGACGG
53	RhaR-1	TTAATCTTTCTGCGAATTGAGATGACGCC
54	LacI <sup>q</sup> -1	GTGAGTCGATATTGTCTTTGTTGACCA
55	Ara-1-intD	GCCTGCATTGCGGCGCTTCAGTCTCCGCTGCATACTGTC CCGTTACCAATTATGACAACCTTGACGG
56	RhaR-1-intD	GCCTGCATTGCGGCGCTTCAGTCTCCGCTGCATACTGTC CTTAATCTTTCTGCGAATTGAGATGACGCC
57	LacI <sup>q</sup> -1-intD	GCCTGCATTGCGGCGCTTCAGTCTCCGCTGCATACTGTC CTTAATAAAGTGAGTCGATATTGTCTTTGTTGACCA
58	FtsZ-1-X	GCCTGCATTGCGGCGCTTCAGTCTCCGCTGCATACTGTC CCGTTACCAATTATGACAACCTTGACGG

5 In like fashion, the *ftsZ* gene was amplified from SEQ ID NO.: 1, 2 and Pta::ftsZ (Garrido, T. et al. 1993. Transcription of *ftsZ* oscillates during the cell cycle of *Escherichia coli*. EMBO J. 12:3957-3965) plasmid and chromosomal constructs, respectively using the following oligonucleotides:

10 For amplification of *araC* through *ftsZ* of SEQ ID NO.: 1 use oligonucleotides:

AraC-1  
FtsZ-2

15 For amplification of *rhaR* through *ftsZ* of SEQ ID NO.: 2 use oligonucleotides:

RhaR-1  
FtsZ-2

20

For amplification of *lacI<sup>q</sup>* through *ftsZ* of Pta::ftsZ (Garrido, T., et al.) use oligonucleotides:

lacI<sup>q</sup>-1  
ftsZ-2

25

WO 03/072014

PCT/US02/16877

The above amplified DNA regions were gel-purified and used as template for the second round of PCR using oligonucleotides containing homology with the E. coli chromosomal gene *intD* and on the other end with random sequence termed "X". Oligonucleotides used in this round of PCR are shown below:

- 5 For amplification of *araC* through *ftsZ* from SEQ ID NO.: 1 to contain homology to *intD* and the random X use oligonucleotides:

AraC-1-*intD*  
FtsZ-1-X

10

For amplification of *rhaR* through *ftsZ* from SEQ ID NO.: 2 to contain homology to *intD* and the random X use oligonucleotides:

- 15 RhaR-1-*intD*  
FtsZ-1-X

- 20 For amplification of *lacIq* through *ftsZ* from *Ptac::ftsZ* to contain homology to *intD* and the random X use oligonucleotides:

LacIq-1-*intD*  
FtsZ-1-X

25

The PCR products from these PCR reactions are as shown below:

*intD* - *araC* - Ara promotor - *ftsZ* - "X"

- 30 *intD* - *rhaRS* - Rha promotor - *ftsZ* - "X"

*intD* - *lacI<sup>q</sup>* - *Ptac* promotor - *ftsZ* - "X"

- 35 To amplify the mature complexes, the following regions were mixed and amplified with the coupled oligonucleotide sequence primers:

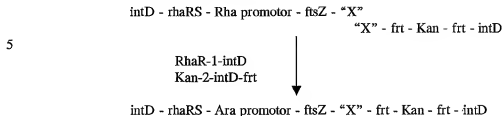
SEQ ID NO.: 3 was produced using:

- 40 *intD* - *araC* - Ara promotor - *ftsZ* - "X"  
"X" - *frt* - Kan - *frt* - *intD*
- AraC-1-*intD*  
Kan-2-*intD*-*frt*
- ↓
- 45 *intD* - *araC* - Ara promotor - *ftsZ* - "X" - *frt* - Kan - *frt* - *intD*

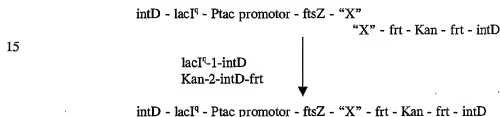
WO 03/072014

PCT/US02/16877

SEQ ID NO.: 4 was produced using:



SEQ ID NO.: 5 was produced using:



These expression constructs may be expressed from the plasmid, placed in single copy, replacing the native ftsZ copy on the E. coli chromosome (Garrido, T., et al. 1993. Transcription of ftsZ oscillates during the cell cycle of Escherichia coli. EMBO J. 12:3957-3965), or in duplicate copy retaining the native ftsZ copy while inserting one of the expression constructs in Table 6 into the intD gene on the same chromosome. Chromosomal duplications were constructed using the RED recombinase system (Katsenko, K. A., and B. L. Wanner. One-Step Inactivation of Chromosomal Genes in Escherichia coli K-12 Using PCR Products. Proc. Natl. Acad. Sci. 97:6640-6645. 2000) and are shown in SEQ ID NO 3-5. The later constructs allow native replication during non-minicell producing conditions, thus avoiding selective pressure during strain construction and maintenance. Furthermore, these strains provide defined points of minicell induction that improve minicell purification while creating conditions that allow strain manipulation prior to, during, and following minicell production. By way of non-limiting example these manipulations may be protein production that the cytoplasmic redox state, modify plasmid copy number, and/or produce chaperone proteins.

For minicell production, a minicell producing strain described in the previous section is grown overnight in Luria broth (LB) supplemented with 0.1% dextrose, 100 µg/ml ampicillin, and when using the single-copy ftsZ construct, 15 µM IPTG. All incubations were performed at 37°C. For minicell induction only, overnight strains are subcultured

WO 03/072014

PCT/US02/16877

1/1000 into the same media. If minicell induction is to be coupled with co-expression of other proteins that are controlled by a catabolite repression-sensitive regulator, dextrose was excluded. Minicell induction is sensitive to aeration and mechanical forces. Therefore, flask size, media volume and shake speed is critical for optimal yields. Likewise, bioreactor conditions must be properly regulated to optimize these production conditions.

In shake-flask cultures, strains are grown to early exponential (log) phase as monitored by optical density (OD) at 600 nm ( $OD_{600}$  0.05-0.20). (Bioreactor conditions may differ significantly depending on the application and yield desired). For minicell induction alone, early log phase cultures are induced with the appropriate inducer concentration shown in Table 6. For coupled co-expression, these cultures are induced as shown in Table 6 for the appropriate minicell regulator, while the coupled protein(s) is induced with the inducer appropriate for the regulator controlling the synthesis of that protein. Cultures are grown under the appropriate conditions and harvested during late log ( $OD_{600}$  0.8-1.2). Depending on the application, minicell induced cultures may be immediately chilled on ice prior to purification, or maintained at room temperature during the harvesting process.

To separate minicells from viable, parental cells, cultures are subjected to differential centrifugation (Voros, J., and R. N. Goodman. 1965. Filamentous forms of *Erwinia amylovora*. *Phytopathol.* 55:876-879). Briefly, cultures are centrifuged at 4,500 rpm in a GSA rotor for 5 min. Supernatants are removed to a fresh bottle and centrifuged at 8,000 rpm for an additional 10 min to pellet minicells. Pelleted minicells (containing contaminating parental cells) are resuspended in 2 ml LB, LBD (LB supplemented with 0.1% dextrose), Min (minimal M63 salt media) (Roozen, K. J., et al. 1971. Synthesis of ribonucleic acid and protein in plasmid-containing minicells of *Escherichia coli* K-12. *J. Bacteriol.* 107:21-23), supplemented with 0.5% casamino acids) or MDT (minimal M63 salt media, supplemented with 0.5% casamino acids, 0.1% dextrose, and thiamine). Resuspended minicells are next separated using linear density gradients. By way of non-limiting example, these gradients may contain sucrose (Cohen A., et al. 1968. The properties of DNA transferred to minicells during conjugation. *Cold Spring Harb. Symp. Quant. Biol.* 33:635-641), ficol, or glycerol. For example, linear sucrose gradients range from 5-20% and are poured in LB, LBD, Minor MDT. Using a SW28 swinging bucket rotor, gradients are centrifuged at 4,500 rpm for 14 min. Banded minicells are removed, mixed with LB, LBD, Minor MDT, and using a JA-20 rotor are centrifuged at 13,000 rpm for 12 min. Following centrifugation, pellets are resuspended in 2 ml LB, LBD, Minor MDT and subjected to a second density gradient. Following the second density separation, banded minicells are

removed from the gradient, pelleted as described, and resuspended in LB, LBD , Minor MDT for use and/or storage.

Purified minicells are quantitated using an OD<sub>600</sub> measurement as compared to a standard curve incorporating LPS quantity, minicell size, and minicell volume. Quantitated minicells mixtures are analyzed for contaminating, viable parental cells by plating on the appropriate growth media (Table 9).

**TABLE 9: MINICELL PURIFICATION AND PARENTAL CELL QUANTITATION**

Purification	Total cells	Total parental cells	MC / PC ratio	Fold-purification
Before	$4.76 \times 10^{11}$	$3.14 \times 10^{11}$	0.25 / 1	-
After	$1.49 \times 10^{11}$	$6.01 \times 10^4$	$2.48 \times 10^6$ / 1	$5.23 \times 10^6$

#### **EXAMPLE 14: PROTOPLAST FORMATION**

In order to allow a membrane receptor to be presented to the outside environment (displayed), minicells are made into protoplasts. In order to make the integral membrane protein receptors in the inner membrane more accessible for ligand binding, the outer membrane and cell wall were removed. The removal of the outer membrane and cell wall from E. coli whole cells and minicells to produce protoplasts was performed essentially according to previously described protocols with a few modifications (Birdsell et al., Production and Ultrastructure of Lysozyme and Ethylenediaminetetraacetate-Lysozyme Spheroplasts of Escherichia coli, J. Bacteriol. 93:427-437, 1967; Weiss et al., Protoplast Formation in Escherichia Coli, J. Bacteriol. 128:668-670, 1976. Both minicells and whole cells were processed the same way.

In brief, the cells were grown to mid-log phase and pelleted at room temperature (minicells were isolated from cultures in mid-log phase). The pellet was washed twice with 10 mM Tris. Following the second wash protoplast production may be performed using two approaches. In the first approach, following the second wash, the cells were resuspended in 100 mM Tris (pH 8.0) that contained 6-20% sucrose and put in a 37°C waterbath (the Tris/sucrose buffer was pre-warmed to 37°C). The volume used to resuspend the cells was determined by the following equation: (volume of cells x OD<sub>450</sub>) / 10 = resuspension volume. After a 1 minute incubation, 2 mg/mL lysozyme was added to a final concentration of 5-100 µg/mL. The samples were then incubated for 12 minutes at 37°C while being

gently mixed. Next, 100 mM EDTA (pH 7) was slowly added over a period of 2.5 minutes (amount of EDTA added = 1/100-1/10 volume of cells) followed by a 10 min incubation at 37 °C. The protoplasts are also diluted from 20% sucrose down to either 10% or 5% sucrose, which facilitates the complete removal of the outer membrane and cell wall. The protoplasts thus generated were separated from the outer membrane and cell wall using a sucrose step gradient. A sucrose step gradient does not have a gradual increase in sucrose percentage; rather, it goes directly from one percent to the other. For example, protoplasts generated from whole cells are loaded on a step gradient that is made from 5% and 15% sucrose. The protoplasts spin through the 15% sucrose but the debris generated when making the protoplasts does not spin through the 15% sucrose. The protoplasts are thus separated from the debris. The second method to prepare protoplasts, following the second wash, 1X 10<sup>9</sup> cells were resuspended with 50 mM Tris, pH 8.0 containing 0.5-50 mM EDTA and 6-20% sucrose. This mixture was incubated at 37°C for 10 min. Following incubation, the mixture was centrifuged at 13,200 RPM in a microcentrifuge for 2 min. After centrifugation, the pellet was resuspended in 50 mM Tris, pH 8.0 containing 5-100 µg/ml lysozyme and 6-20% sucrose. This mixture was incubated at 37°C for 10 min. Following incubation, the mixture was centrifuged at 13,200 RPM in a microcentrifuge for 2 min, resuspended in 50 mM Tris pH 8.0 containing 6-20% sucrose for use.

An alternative method to remove contaminating LPS is to use affinity absorption with an anti-LPS antibody (Cortex). To accomplish this, the anti-LPS antibody was coated on either an activated agarose or sepharose matrix (Sigma) or epoxy-coated magnetic M-450 beads (Dynal). The spheroplast/protoplast mixture was subjected to the antibody coated matrix either in batch or using column chromatographic techniques to remove contaminating LPS. Following exposure, the unbound fraction(s) was collected and re-exposed to fresh matrix. To monitor the efficiency of the protoplasting reaction and LPS removal, three constructs were used (Table 10).

**TABLE 10: PROTOPLAST MONITORING CONSTRUCTS**

Construct	SEQ ID NO	Plasmid	SEQ ID NO	Inducible protein	Inducer
PMPX-5	6	pMPX-32	7	ΔphoA	Rhamnose
PMPX-5	6	pMPX-53	8	phoA	Rhamnose
PMPX-5	6	pMPX-33	9	toxR-phoA	Rhamnose

**TABLE 11. OLIGONUCLEOTIDE PRIMER SEQUENCES FOR TABLE 10 CONSTRUCTS**

SEQ ID NO.:	Primer name	5' to 3' sequence
59	$\Delta$ phoA-1	GCCTGTTCTGGAAAACCGGGCTGCTCAGGG
60	$\Delta$ phoA-2	GCGGCTTTCATGGTGTAGAAGAGATCGG
61	$\Delta$ phoA-1-PstI	CCGCGCTGCAGATGCCTGTTCTGGAAAACCGGGCTGCTCAGGG
62	$\Delta$ phoA-2-XbaI	GCGCCTCTAGATTATTATTTCAGCCCCAGAGCGGCTTTCATGGTGTAGAAGAGATCGG
63	PhoA-1	GTCACGGCCGAGACTTATAGTCGC
64	PhoA-2	GCGGCTTTCATGGTGTAGAAGAGATCGG
65	PhoA-1-PstI	CCGCGCTGCAGATGTCACGGCCGAGACTTATAGTCGC
66	PhoA-2-XbaI	GCGCCTCTAGATTATTATTTCAGCCCCAGAGCGGCTTTCATGGTGTAGAAGAGATCGG
67	T-phoA-1-PstI	CCGCGCTGCAGATGAACCTGGGGAATCGACTGTTTATTCTGATAGCGGCTTACTTCCCCTCGCAGTATTACTGCTCATGCTGTCTGGAAAACCGGGCTGCTCAGGG
68	T-phoA-2-XbaI	GCGCCTCTAGATTATTATTTCAGCCCCAGAGCGGCTTTCATGGTGTAGAAGAGATCGG

Oligonucleotides SEQ ID NOS.:59, 60, 61 and 62 were used to amplify phoA lacking a leader sequence ( $\Delta$ phoA) from the E. coli chromosome. Once amplified, this region was inserted into SEQ ID NO.: 6 using PstI and XbaI to create SEQ ID NO.: 7.

Oligonucleotides SEQ ID NOS.:63, 64, 65 and 66 were used to amplify phoA containing a leader sequence (phoA) from the E. coli chromosome. Once amplified, this region was inserted into SEQ ID NO.: 6 using PstI and XbaI to create SEQ ID NO.: 8.

Oligonucleotides SEQ ID NOS.:59, 60, 67 and 68 were used to amplify phoA lacking a leader sequence ( $\Delta$ phoA) from the E. coli chromosome and form a translational fusion between the transmembrane domain of toxR from Vibrio cholerae. Once amplified, this region was inserted into SEQ ID NO.: 6 using PstI and XbaI to create SEQ ID NO.: 9.

By co-expression of minicells and protein, minicells were prepared that contained cytoplasmic PhoA (pMPX-32 expresses phoA lacking a leader sequence [ $\Delta$ phoA]), periplasmic PhoA (pMPX-53 expresses native phoA that exports to the periplasmic space), or inner membrane-bound PhoA (pMPX-33 expresses phoA lacking a leader sequence fused to the transmembrane domain (TMD) of the toxR gene product from Vibrio cholerae). Using these expressed proteins, the efficiency of minicell protoplasting was monitored (Table 12).

**TABLE 12. EFFICIENCY OF MINICELL PROTOPLAST PREPARATION AND PURIFICATION**

Step	Location <sup>a</sup>	ΔPhoA	PhoA	T-PhoA	LPS total <sup>b</sup>
Minicell	Pellet	100	100	100	100
EDTA/lysozyme	Whole	100	100	100	100
1 <sup>st</sup> Anti-LPS	Pellet	80	0	80	30
2 <sup>nd</sup> Anti-LPS	Pellet	60	0	60	0

- 5      a. Measuring the location of protein being measured using an anti-BAP antibody (Sigma). Pellet refers to the presence of the expressed protein in the low-speed centrifugation pellet. These pellets contain only intact cellular bodies. Whole refers to the reaction mixture prior to low-speed centrifugation.
- b. Measured using a slot-blot apparatus (Bio-Rad) using the anti-LPS antibody (Cortex)

      The data suggests that periplasmic PhoA is lost during the preparation, while both  
10    cytoplasmic and membrane-bound PhoA are retained in a cellular body that lacks LPS.  
      However, during this process ~ 40% of the total minicell content is lost.

#### **EXAMPLE 15:      T7-DEPENDENT INDUCTION OF EXPRESSION**

      Expression from the pCAL-c expression vector is driven from a T7 bacteriophage promoter that is repressed by the LacI gene product. Transcription of the DNA into mRNA,  
15    and subsequent translation of mRNA into proteins, does not occur as long as the LacI repressor is bound to the T7 promoter. However, in the presence of IPTG, the LacI repressor does not bind the T7 promoter. Thus, induction of expression from pCAL-c sequences is dependent on the presence of IPTG. Slightly different protocols were used for the induction of *Escherichia coli* whole and for the induction of minicells. Slight differences  
20    are also present in the protocols for induction of minicells for <sup>35</sup>S-methionine labeling of proteins in contrast to those for the induction of minicells for Western blot analysis. These induction protocols are described below.

      For expression in *E. coli* whole cells, the cells were first grown overnight in 3 mL of LB and antibiotics. The cultures were screened for the presence of the desired expression  
25    element as previously described. Cultures containing the desired expression elements were diluted 1:100 and grown to an OD<sub>600</sub> of between 0.4 to 0.6. The culture size varied depending on the intended use of the cells. IPTG was then added to a final concentration of

200 µg/mL, and the cells were shaken at 30°C for 4 hours. Following the induction, cells were harvested for analysis.

The induction of minicells was carried out as follows. The minicells were diluted in MMM buffer to 1 mL total volume according to the concentration obtained from the isolation  
5 procedure (OD<sub>490</sub> of about 0.5). The cells were then treated with 50 µg/mL of cycloserine for 30 minutes at 37°C to stop whole cell growth. Following the cycloserine treatment the cells were provided with an amino acid, methionine, which the MMM buffer does not contain. For <sup>35</sup>S-labeled protein induction <sup>35</sup>S-methionine was added to the minicell sample whereas, for unlabeled protein induction unlabeled methionine was added. Fifteen (15) µCi  
10 of <sup>35</sup>S-methionine (Amersham Pharmacia Biotech, Piscataway, NJ) was added to the samples for radiolabeling and 5 µmol of methionine was added to the non-labeled minicell samples. Two hundred (200) µg/mL IPTG was also added to the minicell samples, which were then shaken at 30°C for about 4 hours. Following induction, the minicells were harvested for further preparation or analysis.

#### 15 **EXAMPLE 16: WESTERN BLOT ANALYSIS**

The CBP detection kit was purchased from Stratagene. SDS running buffer, 10% Tris-HCl ready gels, Kaleidoscope Pre-stained Standards, and Laemmli Sample Buffer were purchased from BIO RAD (Hercules, CA). GFP (FL) HRP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Edg-3CT antibody an antibody directed to the  
20 carboxy terminus of was purchased from Exalpha Biologicals (Boston, MA). Anti-6xHis antibody, positrope, and the WesternBreeze Kit were purchased from Invitrogen (Carlsbad, CA). Protocols were carried out essentially according to the manufacturer's instructions unless otherwise indicated.

Three different Western blot protocols were used to detect protein expression in both  
25 a minicell expression system and in a whole cell expression system. For both systems, the SDS-PAGE gel and the transfer protocols were essentially as follows. The samples were denatured by diluting the samples 1:1 in Laemmli buffer (BIORAD) and then sonicated for 10 min. The denatured samples were loaded onto a 10% Tris-Glycine gel (BIORAD) and electrophoresed at 130 V for about 1.5 hours in 1X SDS running buffer (BIORAD). The  
30 electrophoresed proteins were electrotransferred to nitrocellulose membranes at 0.5 Amps for 1.5 hours in Transfer Buffer (5.8 g Tris, 2.9 g glycine, 200 mL methanol, and 3.7 mL of

WO 03/072014

PCT/US02/16877

10% SDS). The nitrocellulose membranes comprising the transferred proteins were used for Western blotting.

GFP Western blots were carried out as follows. The nitrocellulose membrane was blocked for 2 hours with 5% milk in PBST (PBS buffer with 0.05% Tween). Following the blocking step the nitrocellulose membrane was washed twice with PBST. For the detection of GFP protein, an anti-GFP-HRP conjugated antibody (Santa Cruz Biotechnology) was used at a dilution of 1:3000 in PBST (HRP, horse radish peroxidase). The nitrocellulose membrane was incubated in the anti-GFP-HRP antibody solution for one hour and then washed twice with PBST. GFP proteins on the nitrocellulose membrane were detected and visualized using the ECL system (Amersham).

The His-tagged Edg-1 and Edg-3 proteins were detected using a mouse anti-6xHis antibody from Invitrogen and the WesternBreeze chemoluminescent Kit (Invitrogen). The antibody was diluted 1:4000 in buffers provided by the WesternBreeze Kit. The WesternBreeze immunoblot was carried out essentially according to the manufacturer's protocol. The Edg-1-CBP and GFP-CBP fusion proteins were detected using the CBP detection Kit (Stratagene). All antibodies and substrates were provided in the Kit. Figure 3 is a photo of the Western hybridization results showing the presence of Edg-1-6xHis and Edg-3-6xHis in minicells and parent cells.

#### EXAMPLE 17: METHODS TO INDUCE EXPRESSION

Expression in minicells may proceed following purification of minicells and/or minicell protoplasts from parental cells and LPS constituents, respectively. However, for some applications it is suitable to co-express proteins of interest with minicell induction. For these approaches, one may use the protocol described in EXAMPLE 13 for expression of the *phoA* constructs. By way of non-limiting example, either of these approaches may be accomplished using one or more of the following expression constructs (Table 13).

TABLE 13: EXPRESSION CONSTRUCTS

Plasmid	Regulatory element(s)	inducer	Plasmid	SEQ ID NO.:
pMPX-5	<i>rhaRS</i>	Rhamnose	pUC-18	6
pMPX-7	<i>uidR</i>	$\beta$ -glucuronate	pUC-18	10

WO 03/072014

PCT/US02/16877

Plasmid	Regulatory element(s)	inducer	Plasmid	SEQ ID NO.:
pMPX-8	melR	Melibiose	pUC-18	11
pMPX-18	araC	Arabinose	pUC-18	12
pMPX-6	araC	Arabinose	pUC-18	13

**TABLE 14: OLIGONUCLEOTIDE PRIMER SEQUENCES FOR TABLE 13 CONSTRUCTS**

SEQ ID NO.:	Primer name	5' to 3' sequence
69	Rha-1	GCGAATTGAGATGACGCCACTGGC
70	Rha-2	CCTGCTGAATTTCATTAACGACCAG
71	Rha-1-HindIII	CGCGGAAGCTTAATTAATCTTCTGCGAATTGAG ATGACGCCACTGGC
72	Rha-2-PstI	CGCCGTAATCGCCGCTGCGAATGTGATCTGCT GAATTCATTAACGACCAG
73	Uid-1	CGCAGCGCTGTTCTTTGCTCG
74	Uid-2	CCTCATTAAGATAATAACTGG
75	Uid-1-HindIII	GCCGCAAGCTTCGCGAGCGTGTCTTCTGCTCG
76	Uid-2-PstI	CCAATGCATTGGTTCTGCAAGGACTCCTCATTAAG ATAATAATACTGG
77	Mel-1	CGTCTTTAGCCGGGAAACG
78	Mel-2	GCAGATCTCTGGCTTGC
79	Mel-1-HindIII	GCCGCAAGCTTCGCTCTTTAGCCGGGAAACG
80	Mel-2-SalI	CGGTCGACGCAGATCTCTCTGGCTTGC
81	Ara-1	CAAGCCGTCAATTGTCTGATTCC
82	Ara-2	GGTGAATTCTCTGCTAGCCC
83	Ara-1-HindIII	GCGCCAAGCTTCAAGCCGTCAATTGTCTGATTCC
84	Ara-2-PstI	CTGCAGGGTGAATCTCTCTGCTAGCCC
85	Ara-1-XhoI	GCTTAAGCTCGAGCTTAATAACCAAGCCGTCAATTG TCTGATTCT
86	Ara-2-SstI	GCTTAACCGCGGGCCAAGCTTGCATGCCTGCTCC

5

Oligonucleotides SEQ ID NOS.:69, 70, 71 and 72 were used to amplify the rhaRS genes and their divergent control region from the E. coli chromosome. Once amplified, this region was inserted into pUC18 using HindIII and PstI to create SEQ ID NO.: 6.

Oligonucleotides SEQ ID NOS.:73, 74, 75 and 76 were used to amplify the uidR control region, the uidR gene and the control region for expression from the E. coli chromosome. Once amplified, this region was inserted into pUC18 using HindIII and PstI to create SEQ ID NO.: 10.

WO 03/072014

PCT/US02/16877

Oligonucleotides SEQ ID NOS.:77, 78, 79 and 80 were used to amplify the melR gene and its divergent control region from the E. coli chromosome. Once amplified, this region was inserted into pUC18 using HindIII and SalI to create SEQ ID NO.: 11.

5 Oligonucleotides SEQ ID NOS.:81, 82, 83 and 84 were used to amplify the araC gene and its divergent control region from the E. coli chromosome. Once amplified, this region was inserted into pUC18 using HindIII and PstI to create SEQ ID NO.: 12.

Oligonucleotides SEQ ID NOS.:81, 82, 85 and 86 were used to amplify the araC gene and its divergent control region was PCR amplified from pBAD-24. Once amplified, this region was inserted into pEGFP (Clontech) using XhoI and SstI to create SEQ ID NO.:  
10 13.

Except of pMPX-6, these expression constructs contain the same multiple cloning site. Therefore, any protein of interested may be inserted in each modular expression construct for simple expression screening and optimization.

By way of non-limiting example, other proteins that may be expressed are listed in  
15 Table 15.

TABLE 15: OTHER EXPRESSED PROTEINS

Protein	Origin	Construct	Purpose	SEQ ID NO.:
Edg3	Rat	native	GPCR	14
$\beta$ 2AR	Human	native	GPCR	15
TNFR-1a (human)	Human	residues 29-455	Receptor	18
TNFR-1b (human)	Human	residues 41-455	Receptor	17
TNF (human)	Human	native	Gene transfer	19
T-EGF	Human	chimera	Gene transfer	20
T-Invasin	Y. pseudotuberculosis	chimera	Gene transfer	21

WO 03/072014

PCT/US02/16877

TABLE 16: OLIGONUCLEOTIDE PRIMER SEQUENCES FOR TABLE 15

SEQ ID NO.:	Primer name	5' to 3' sequence
87	Edg-1	GGCAACCACGCACGCGCAGGGCCACC
88	Edg-2	CAATGGTGATGGTGATGATGACCGG
89	Edg-1-SalI	CGCGGTCGACATGGCAACCACGCACGCGCAGG GCCACC
90	Edg-2-KpnI	GCGCCGGTACCTTATCAATGGTGATGGTGATG ATGACCGG
91	$\beta$ 2AR-1	GGGGCAACCCGGGAACGGCAGCGCC
92	$\beta$ 2AR-2	GCAGTGAGTCATTGTACTACAATTCTCC
93	$\beta$ 2AR-1-SalI	CGCGGTCGACATGGGGCAACCCGGGAACGGCA GCGCC
94	$\beta$ 2AR-2-BamHI	GCGCCGGATCCITATTATAGCAGTGAGTCATT GTACTACAATTCTCTCC
95	TNFR(29)-1	GGACTGGTCCCTCACC TAGGGGACAGGG
96	TNFR(29)-2	CTGAGAAGACTGGGCGCGGGCGGGAGG
97	TNFR(29)-1-SalI	CGCGGGTCGACATGGGACTGGTCCCTCACCTA GGGGACAGGG
98	TNFR(29)-2-KpnI	GCGCCGGTACCTTATTACTGAGAAGACTGGGC GCGGGCGGGAGG
99	TNFR(41)-1	GATAGTGTGTGTCCCC
100	TNFR(41)-2	CTGAGAAGACTGGGCGC
101	TNFR(41)-1-NcoI	GGGAGACCATGGATAGTGTGTGTCCCC
102	TNFR(41)-2-XbaI	GCCTCATCTAGATTACTGAGAAGACTGGGCGC
103	TNF-1	GAGCACTGAAAGCATGATCCGGGACG
104	TNF-2	CAGGGCAATGATCCCAAAGTAGACCTGC
105	TNF-1-EcoRI	CCGCGGAATTCATGAGCACTGAAAGCATGATC CGGGACG
106	TNF-2-HindIII	GGCGCAAGCTTATCACAGGGCAATGATCCAA AGTAGACCTGC
107	T-EGF-1	TCTGATAGCGGTCTTACTTCCCCTCGCAGTATT ACTGCTCAATAGTGACTCTGAATGTCCCCTGT CCACGATGGGTACTGCCTCCATGATGGTGTGT GCATGTATATTG
108	T-EGF-2	AGGTCTCGGTACTGACATCGCTCCCCGATGTA GCCAACAAACAGTTGCATGCATACTTGTCCA ATGCTTCAATATACATGCACACACCATCATGG AGGCA
109	T-EGF-3	CCGCGGGTACCATGAACCTGGGGAATCGACTG TTTATTCTGATAGCGGTCTTACTTCCCCTCG
110	T-EGF-4	GCGCCAAGCTTATTAGCGCAGTCCCACCACT TCAGGTCTCGGTACTGACATCGCTCCCCG
111	Inv-1	TCATTACATTGAGCGTCACCG
112	Inv-2	TTATATTGACAGCGCACAGAGCGG
113	Inv-1-ToxR-EcoRI	GCAAGAATTACCATGAACCTGGGGAATCGAC TGTTTATTCTGATAGCGGTCTTACTTCCCCTCG CAGTATTACTGCTCTCATTACATTGAGCGTCA CCG

WO 03/072014

PCT/US02/16877

SEQ ID NO.:	Primer name	5' to 3' sequence
114	Inv-2-PstII	CGCGGTTACGTAAGCAACTGCAGTTATTTGA CAGCGCACAGAGCGG

Oligonucleotides SEQ ID NOS.:87, 88, 89 and 90 were used to amplify rat Edg3 from rat cDNA. Once amplified, this region was inserted into SEQ ID NO.: 6 (pMPX-5) using SalI and KpnI to create SEQ ID NO.:14.

- 5 Oligonucleotides SEQ ID NOS.:91, 92, 93 and 94 were used to amplify human  $\beta$ 2 adrenergic receptor ( $\beta$ 2AR) from human heart cDNA. Once amplified, this region was inserted into SEQ ID NO.: 6 (pMPX-5) using SalI and BamHI to create SEQ ID NO.:15.

- 10 Oligonucleotides SEQ ID NOS.:95, 96, 97 and 98 were used to amplify human tumor necrosis factor receptor (TNFR residues 29-455) from human Jurkat CL71 cDNA. Once amplified, this region was inserted into SEQ ID NO.: 12 (pMPX-18) using SalI and KpnI to create SEQ ID NO.:18.

- 15 Oligonucleotides SEQ ID NOS.:99, 100, 101 and 102 were used to amplify human tumor necrosis factor receptor (TNFR residues 41-455) from human Jurkat CL71 cDNA. Once amplified, this region was inserted into pBAD24 using NcoI and XbaI to create SEQ ID NO.:17.

- 20 Oligonucleotides SEQ ID NOS.:103, 104, 105 and 106 were used to amplify human tumor necrosis factor (TNF) from human Jurkat CL71 cDNA. Once amplified, this region was inserted into SEQ ID NO.: 13 (pMPX-6) using EcoRI and HindIII to create SEQ ID NO.:19.

**TABLE 17: PROGRAM TO ANNEAL GRADIENT PCR WITH PFX POLYMERASE**

Step	Temp (°C)	Time (min)
1	95	2.0
2	95	0.5
3	64	0.5
4	68	2.5
5	Goto 2, 2X	
6	95	0.5
7	62	0.5
8	68	2.5
9	Goto 6, 4X	
10	95	0.5
11	60	0.5
12	68	2.5

WO 03/072014

PCT/US02/16877

Step	Temp (°C)	Time (min)
13	Goto 10, 6X	
14	95	0.5
15	58	0.5
16	68	2.5
17	Goto 14, 24X	
18	4	hold
19	end	

Oligonucleotides SEQ ID NOS.:107, 108, 109 and 110 were mixed and PCR amplified using anneal gradient PCR (Table 17) to form mature human epidermal growth factor (EGF) (residues 971-1023) translationally fused to the transmembrane domain of toxR from *Vibrio cholerae*. Once amplified, this region was inserted into SEQ ID NO.: 13 (pMPX-6) using KpnI and HindIII to create SEQ ID NO.:20.

Using PFX polymerase (Invitrogen) oligonucleotide SEQ ID NO.:111, 112, 113 and 114 were used to amplify invasin residues 490-986 (inv) from *Yersinia pseudotuberculosis* chromosomal DNA and form a translational fusion between the transmembrane domain of toxR from *Vibrio cholerae*. Once amplified, this region was inserted into SEQ ID NO.:13 (pMPX-6) using EcoRI and PstI to create SEQ ID NO.:21.

These proteins were proof-of-principle constructs used to evaluate the minicell platform. For purposes of this initial evaluation, all proteins except TNF, T-EGF and T-Invasin were cloned into pMPX-5, with these later proteins cloned into pMPX-6 for gene transfer experiments.

Whether the approach for protein expression is co-expression with minicell induction or expression following minicell and/or protoplast isolation, the procedure to transform the expression constructs is the same. To accomplish this, protein constructs were initially cloned into *E. coli* MG1655 and then into the minicell producing strain of interest. Transformation events were selected prior to minicell induction. For co-induction of protein and minicells, see the protocol for phoA expression above. For post-minicell and/or protoplast purification induction experiments, following minicell purification and/or protoplast preparation and purification, these cellular bodies were induced for protein production in either LBD or MDT at a minicell or protoplast / volume ratio of  $1 \times 10^9$  minicells or protoplasts / 1 ml media. Media was supplemented with the appropriate inducer concentration (see Table 6). Protein induction is sensitive to a variety of factors including, but not limited to aeration and temperature, thus reaction volume to surface area ratio is important, as is the method of shaking and temperature of induction. Therefore, each protein must be treated as required to optimize expression. In addition to expression parameters,

protoplasted minicells are sensitive to osmotic and mechanical forces. Therefore, protoplast protein induction reactions must also contain 10% sucrose with greater volume to surface area ratios than required for intact minicells to achieve similar aeration at lower revolutions.

Using the T-PhoA as a non-limiting example, protein expression was performed during and following minicell isolation. To accomplish this task, t-phoA co-expressed with minicell induction was compared to t-phoA expressed after minicell isolation. In both cases, overnight minicell-producing parental strains containing pMPX-5::t-phoA were subcultured into LBD supplemented with the appropriate antibiotic. Cultures were grown to OD<sub>600</sub> 0.1 and induced for minicell production alone or for both minicell and protein production. Both cultures were harvested at OD<sub>600</sub> 1.0 and minicells produced were harvested as described above. Minicells to be induced for T-phoA production following purification were induced by introducing  $1 \times 10^9$  purified minicells into a 15 ml culture tube containing 1 ml MDT with 1 mM L-rhamnose. Minicell protein induction was allowed to proceed for up to 14 hours and compared to protein production obtained using the co-expression approach. For each approach, minicells were fractionated and analyzed for membrane association, total protein, and membrane association-dependent enzymatic activity. These observations were compared to post-induction, pre-isolation parental cell/minicell (PC/MC) mixtures from the co-expressed reactions. The first observation was that co-expression of minicell and protein induction was superior to post-minicell purification induction (Table 18). However, although the kinetics are slower for the post-minicell purification induction protocol, the end result is equivalent.

**TABLE 18. COMPARATIVE EXPRESSION: CO-EXPRESSION VERSUS POST MINICELL PURIFICATION INDUCTION**

Time of induction	Purified minicell induction <sup>a</sup>	Co-expression induction <sup>a</sup>
1.0	8.0	-
2.0	-	812.2
4.0	70.0	-
14.0	445.0	-

a. Nanogram expressed T-PhoA per  $1 \times 10^9$  minicells.

WO 03/072014

PCT/US02/16877

Using the co-expression induction procedure, the amount of membrane-associated T-PhoA was measured and compared for both parental cells and minicells. Briefly, following co-expression induction of T-PhoA and minicells, minicells were purified and their membranes isolated. For membrane isolation, minicells containing expressed T-PhoA were subjected to three rounds of freeze-thaw lysis in the presence of 10 µg/ml lysozyme. Following freeze-thaw cycling, the reaction was subjected to sonication. Sonicated material was centrifuged at 6,000 rpm in a microcentrifuge for 5 min at room temperature. Supernatants were transferred to a fresh 1.5 ml Eppendorf tube and centrifuged at 70,000 rpm using a TLA-100 rotor. Following centrifugation, the pellet was resuspended in buffer and analyzed for total T-PhoA protein (Table 19) and T-PhoA enzyme activity (Table 20).

**TABLE 19: MEMBRANE ASSOCIATED T-PHOA: PARENTAL CELLS VERSUS MINICELLS**

Cell type <sup>a</sup>	Protein total <sup>a</sup>	T-PhoA total <sup>b</sup>	T-PhoA % total	Protein membrane associated <sup>a</sup>	T-PhoA membrane associated <sup>b</sup>	T-PhoA % membrane protein total
Parental cells	107.5	5.3	4.9	10.7	3.1	29.0
Minicells	4.6	0.8	17.5	1.0	0.5	50.0
Minicells EQ <sup>b</sup>	25.2	4.4	-	5.5	2.7	-

- a. Total protein as determined by BCA assay (Pierce)
- b. Microgram expressed T-PhoA per 1 X 10<sup>9</sup> minicells as determined via Western using an anti-PhoA antibody (Sigma) versus a PhoA standard curve (BCA determined).
- c. Equivalent membrane lipid to parental cell

**TABLE 20: PHOA ENZYMATIC ACTIVITY<sup>a</sup> (RELATIVE UNITS): PARENTAL CELLS VERSUS MINICELLS.**

Cell type <sup>b</sup>	Unlysed	Lysed, total	Lysed, membrane
Parent cell	-	358	240
Minicell	275	265	211
Minicell EQ <sup>c</sup>	1,504	1,447	1,154

- a. Activity determined colorimetrically using PNPP measuring optical density at 405 nm

- b. Based on  $1 \times 10^9$  parental cells or minicells per reaction
- c. Equivalent membrane lipid to parental cell

These results suggest that co-expression induction of T-PhoA and minicells together  
5 results in minicells containing an equivalent amount of T-PhoA produced in both parental  
cells and minicells. However, the percent of T-PhoA compared to total protein is 3.5X  
greater in minicells than in parental cells. Furthermore, of the protein made, T-PhoA  
constitutes 50% of the total membrane protein in minicells, whereas it is only 29% in  
parental cells. It should be noted that the T-PhoA protein associated with the membrane can  
10 be easily removed by treatment with mild, non-ionic detergent suggesting that the T-PhoA  
present in the membrane pellet is indeed associated with the membrane and not an insoluble,  
co-sedimenting precipitate (data not shown). Finally, PhoA is a periplasmic enzyme that  
requires export to the periplasmic space for proper folding and disulfide bond formation.  
Both of which are required for enzymatic activity. In the time course of this experiment,  
15 expression of  $\Delta$ PhoA lacking a leader sequence does not demonstrate enzymatic activity.  
Furthermore, there is no difference between unlysed and lysed minicells containing expressed  
T-PhoA (Table 20) also demonstrating that the PhoA enzyme domain of the T-PhoA chimera  
must be present in the periplasmic space. Therefore, the T-PhoA construct must membrane  
associate and the PhoA domain must orient into the periplasmic space for enzymatic activity.  
20 Thus, when comparing equivalent amounts of membrane lipid between parental cells and  
minicells in Table 20, membrane association-dependent T-PhoA activity is almost 5X greater  
than in parental cells. Taking into account the data in Table 19 where 50% of T-PhoA is in  
the membrane compared to 29% in parental cells, the difference in T-PhoA membrane  
association is not sufficient to explain the almost 5X increase in minicell activity. These  
25 observations suggest that minicells contain a capacity to support more expressed membrane  
protein than parental cells and that the protein that associates with the membrane is more  
active. This activity may be simply result from minicells allowing greater efficiency of  
folding and disulfide bond formation for this particular protein. However, do to the fact that  
minicells do not contain chromosome, it is also possible that the overexpression of this  
30 protein is readily finding membrane-binding sites in the absence of chromosomally produced  
competitors present in parental cells. Furthermore, overexpression of proteins often leads to  
increased protease expression. Because minicells do not contain chromosome, these  
otherwise degraded surplus T-PhoA is allowed the continued opportunity to insert and

WO 03/072014

PCT/US02/16877

properly fold in the membrane, an attribute that could lend favor to overexpression of more complex membrane proteins.

**EXAMPLE 18: EXEMPLARY METHODS TO INDUCE AND STUDY COMPLEX MEMBRANE PROTEINS**

5 Expression of non-native (exogenous) complex membrane proteins in bacterial systems can be difficult. Using the minicell system, we are able to eliminate toxicity issues. However, issues still remain with proper translation, compartmentalization at the membrane, insertion in the membrane and proper folding for native activity. To account for these potential problems we have constructed a modular chimeric system that incorporates leader sequences and chaperone-recognized soluble domains that are native to our bacterial minicell 10 system. In addition, we created modular constructs that overexpress the native chaperones groESL and trigger factor (tig). Finally, we have constructed minicell-producing strains that contain mutations that effect protein export and disulfide bond formation. For non-limiting examples of these constructs see Table 21.

15

**TABLE 21: NON-LIMITING TOOLS FOR EXOGENOUS COMPLEX PROTEIN SYNTHESIS AND FUNCTION**

Tool	Ref.	Residues of sequence	Purpose	SEQ ID NO
pMPX-5::phoA leader	-	1-48	Membrane targeting	22
pMPX-5::phoA leader	-	1-494	Membrane targeting	23
pMPX-5::malE leader	1	1-28	Membrane targeting	24
pMPX-5::malE leader	1	1-370	Membrane targeting	25
pMPX-17 (groESL, tig)	-	-	Chaperone	26
pMPX-5::trxA::FLAG	2	2-109 <sup>a</sup>	Solubility	27

a. Residues do not include FLAG sequence.

20

References to Table 21.

1. Grishammer, R., et al. 1993. Expression of a rat neurotensin receptor in Escherichia coli. *Biochem. J.* 295:571-576.
2. Tucker, J., and R. Grishammer. 1996. Purification of a rat neurotensin receptor expressed in Escherichia coli. *Biochem. J.* 317:891-899.

25

**TABLE 22: OLIGONUCLEOTIDE PRIMER SEQUENCES FOR TABLE 21 CONSTRUCTS**

SEQ ID NO.:	Primer name	5' to 3' sequence
115	PhoA lead-1	GTCACGGCCGAGACTTATAGTCGC
116	PhoA lead-2	GGTGTCCGGGCTTTTGTACAGG
117	PhoA lead-1-PstI	CGCGGCTGCAGATGTCACGGCCGAGACTTATAGTCGC
118	PhoA lead-2-XbaI	CGCGGTCTAGATTCTGGTGTCGGGCTTTTGTACAGG
119	PhoA complete	CAGCCCCAGAGCGGCTTTCATGG
120	PhoA complete-2-XbaI	CGCGGTCTAGATTTCAGCCCCAGAGCGGCTTTCATGG
121	MalE lead-1	CGCGGCTGCAGATGAAAAATAAAACAGGTGCA CGCATCTCTCGCATTATCCGCATTACGACGATG ATGTTTTCCGCGCTCGGCTCTGCCAAAATCTCT AGACGCGG
122	MalE lead-2	CCGCGTCTAGAGATTTTGGCGAGAGCCGAGGC GAAAAACATCATCGTTCGTTAATGCGGATAATG CGAGGATGCGTGACCTGTTTTTATTTCATCT GCAGCCGCG
123	MalE-1	GGTGACAGCATCTCTCGCATTATCCGC
124	MalE-2	CGGCATACCAGAAAGCGGACATCTGC
125	MalE-1-PstI	CGCGGCTGCAGATGAAAAATAAAACAGGTGCA CGCATCTCTCGCATTATCCGC
126	MalE-2-XbaI	CGCGGTCTAGAACGCACGGCATAACCAGAAAGC GGACATCTGC
127	Tig-1	CGCGACAGCGCGCAATAACCGTTCTCG
128	Tig-2	GCTGGTTATCAGCTCGTTGAAAGTGG
129	Tig-1-NarI	GCGCGGGCGCCATACGCGACAGCGCGCAATAA CCGTTCTCG
130	Tig-2-XbaI	GGCGCTCTAGATTATTATTACGCCTGCTGGTTC ATCAGCTCGTTGAAAGTGG
131	Gro-1	GGTAGCACAATCAGATTGCTTTATGACGG
132	Gro-2	GCCGCCCATGCCACCCATGCCGCC
133	Gro-1-XbaI	GCGTCTAGAGGTAGCACAATCAGATTGCTTAT GACGG
134	Gro-2-HindIII	GGCGCAAGCTTATTATTACATCATGCCCCCAT GCCACCCATGCCGCC

WO 03/072014

PCT/US02/16877

SEQ ID NO.:	Primer name	5' to 3' sequence
135	TrxA-1	GCGATAAAATTATTACCTGACTGACG
136	TrxA-2	GCGTCGAGGAACCTCTTCAACTGACC
137	TrxA-1-Fxa-PstI	CGCGGCTGCAGATGATCGAAGCCCGCTCTAGACTCGAGAGCGATAAAATTATTACCTGACTGACG
138	TrxA-2-FLAG-BamHI	CCGCGGGATCCTTATTAATCATCATGATCTTTA TAATCGCCATCATGATCTTTATAATCCTCGAGC GCCAGGTTAGCGTCGAGGAACCTCTTCAACTGACC

Oligonucleotides SEQ ID NOS.:115, 116, 117 and 118 were used to amplify the *phoA* leader (residues 1-49) from *E. coli* chromosomal DNA. Once amplified, this region was inserted into SEQ ID NO.: 6 (pMPX-5) using PstI and XbaI to create SEQ ID NO.:22.

- 5 Oligonucleotides SEQ ID NOS.:115, 117, 119 and 120 were used to amplify the complete *phoA* gene from *E. coli* chromosomal DNA. Once amplified, this region was inserted into SEQ ID NO.: 6 (pMPX-5) using PstI and XbaI to create SEQ ID NO.23.

- Oligonucleotides SEQ ID NOS.:121 and 122 were used to construct the *malE* leader (residues 1-28) sequence. Once annealed, this construct was inserted into SEQ ID NO.: 6 (pMPX-5) using PstI and XbaI to create SEQ ID NO.:24.

Oligonucleotides SEQ ID NOS.:123, 124, 125 and 126 were used to amplify the *malE* expanded leader (residues 1-370) from *E. coli* chromosomal DNA. Once amplified, this region was inserted into SEQ ID NO.: 6 (pMPX-5) using PstI and XbaI to create SEQ ID NO.:25.

- 15 Oligonucleotides SEQ ID NOS.:127, 128, 129 and 130 were used to amplify the *tig* control and gene region from *E. coli* chromosomal DNA. Once amplified, this region was ligated to the *groESL* amplified region below using XbaI prior to insertion into SEQ ID NO.: 6 (pMPX-5) using NarI (from the *tig* region) and HindIII (from the *groESL* region) to create SEQ ID NO.:26.

- 20 Oligonucleotides SEQ ID NOS.:131, 132, 133 and 134 were used to amplify the *groESL* control and gene region from *E. coli* chromosomal DNA. Once amplified, this region was ligated to the *tig* amplified region above using XbaI prior to insertion into SEQ ID NO.: 6 (pMPX-5) using NarI (from the *tig* region) and HindIII (from the *groESL* region) to create SEQ ID NO.:26.

Oligonucleotides SEQ ID NOS.:135, 136, 137 and 138 were used to amplify trxA (residues 2-109) from *E. coli* chromosomal DNA and insert FLAG and Factor Xa sequences. Once amplified, this region was inserted into SEQ ID NO.: 6 (pMPX-5) using PstI and BamHI to create SEQ ID NO.:27.

5 By way of non-limiting example, the pMPX-5::phoA leader (residues 1-48), pMPX-5::phoA leader (residues 1-494), pMPX-5::malE leader (residues 1-28), and pMPX-5::malB leader (residues 1-370) constructs are designed to direct expressed exogenous membrane proteins to the minicell cytoplasmic membrane. In addition to these constructs, By way of non-limiting example, mutations in *E. coli* genes *secA* and *secY*, specifically mutation prlA4  
10 (Strader, J., et al. 1986. Kinetic analysis of lamB mutants suggests the signal sequence plays multiple roles in protein export. *J. Biol. Chem.* 261:15075-15080), permit promiscuous targeting to the membrane. These mutations, like the above constructs are integrated into the minicell expression system. To complement these mutations, the chaperone complex groESL and trigger factor have also been incorporated into the expression system. By way of non-  
15 limiting example, pMPX-5::trxA::FLAG will be used to create a carboxy-terminal fusion to the protein of interest to increase the membrane insertion efficiency of the membrane protein of interest (Tucker, J., and R. Grishammer. 1996. Purification of a rat neurotensin receptor expressed in *Escherichia coli*. *Biochem. J.* 317:891-899). Also By way of non-limiting example, pMPX-5::FLAG::toxR and pMPX-5::FLAG:: $\lambda$ C1 constructs will be  
20 prepared to create a carboxy-terminal fusion to the protein of interest for use in a reporter-based assay for protein-protein interactions. By way of non-limiting example, the protein of interest for this system is a GPCR. Also By way of non-limiting example, this GPCR may be the neurotensin receptor from rat (Grishammer, R., et al. 1993. Expression of a rat neurotensin receptor in *Escherichia coli*. *Biochem. J.* 295:571-576.), or the  $\beta$ 2 adrenergic  
25 receptor from humans (Freissmuth, M., et al. 1991. Expression of two  $\beta$ -adrenergic receptors in *Escherichia coli*: functional interaction with two forms of the stimulatory G protein. *Proc. Natl. Acad. Sci.* 88:8548-8552). Insertion of a GPCR into one of these reporter constructs creates a carboxy-terminal fusion between the GPCR of interest and the DNA-binding regulatory domain of the ToxR positive activator, the  $\lambda$ C1 repressor, or the  
30 AraC positive activator. To complete this reporter system, By way of non-limiting example pMPX-5::(X)::toxR or pMPX-5::(X):: $\lambda$ C1 will be used to create a carboxy-terminal fusion to the protein of interest for use in a reporter-based assay for protein-protein interactions, where (X) may be any protein or molecule involved in an intermolecular or intramolecular interaction. By way of non-limiting example, this molecule of interest may be a G-protein.

### MINICELLS OR MINICELL PROTOPLASTS

between a minicell and a mammalian cell in vitro, or in vivo, and this gene transfer may

occur through cell-specific interactions, through general interactions, or a combination of each. To accomplish this task three basic constructs were created. Each of these constructs is created in pMPX-6 which contains a CMV promotor controlling the synthesis of GFP. The plasmid pMPX-6 was constructed by cloning the araC through the multiple cloning site of pBAD24 into pEGFP (Clontech). This construct provided a bacterial regulator as well as a method to monitor the success of gene transfer using GFP expression from the CMV promotor. In design, the protein expressed using the bacterial promotor will drive the cell-cell interaction, while the successful transfer of DNA from the minicell to the recipient cell will initiate the production of GFP. By way of non-limiting example, proteins that will drive the cell-cell interaction may be the invasins from *Yersinia pseudotuberculosis*, which stimulates  $\beta 1$  integrin-dependent endocytic events. To properly display the invasins on the surface of minicells, the domain of invasins that stimulates these events (residues 490-986) (Dersch, P., and R. R. Isberg. 1999. A region of the *Yersinia pseudotuberculosis* invasion protein enhances integrin-mediated uptake into mammalian cells and promotes self-association. *EMBO J.* 18:1199-1213) was fused to the transmembrane domain of ToxR. Expression of this construct from pMPX-6 will display T-Inv on the surface of the minicell and stimulate endocytosis with any cell displaying a  $\beta 1$  integrin. Thus, T-Inv display will provide a general mechanism of gene transfer from minicells. To provide specificity, By way of non-limiting example, the ligand portion of epidermal growth factor (EGF) may be fused to the transmembrane domain of ToxR, thus creating a protein that will interact with cells displaying the EGF receptor (EGFR). Likewise, tumor necrosis factor (TNF) may also serve this purpose by stimulating cell-cell interactions between minicells displaying TNF and cells displaying TNF receptor (TNFR). Although EGF-EGFR and TNF-TNFR interactions may stimulate cell-cell fusion between minicells and recipient cells, or minicell uptake, this alone may not be sufficient to efficiently transfer genetic information from minicells. Therefore, a genetic approach to increasing the cell-cell genetic transfer may be the development of a genetic switch that senses the specificity interaction, e.g. EGF-EGFR interaction, and turns on the production of a second gene product, e.g. invasins, that stimulates the endocytic event. By way of non-limiting example, this genetic switch may be similar to the GPCR-G-protein interaction reporter system above, in that an extracellular event stimulates the dimerization of a transcriptional active regulator, thus turning on the production of invasins or invasins-like protein. In either approach, the display system to stimulate transfer of genetic information from minicells to recipient cells may also be applicable to the transfer of substances other than genetic information, e.g. pre-synthesized therapeutic drugs.

To test this targeting methodology, different pMPX-6 constructs containing each of these general or specific cell-cell interaction proteins will be transformed into a minicell producing strain and either by co-expression induction of minicells, by post-minicell purification induction, or by post-protoplasting induction, minicells displaying the targeting protein of interest will be produced. When using the co-expression induction and post-minicell purification induction of the targeting protein approaches, it is necessary to protoplast the purified minicells after protein induction. Once the targeting protein has been displayed on the surface of a minicell protoplast, these protoplasts are ready to be exposed to target cells. For preliminary experiments these interactions will be monitored using cell culture of Cos cells in comparison to lipofectamine (Invitrogen), electroporation, and other transfection techniques. Initial experiments will expose protoplasts displaying T-Inv to Cos cells and compare the transfection efficiency to protoplast containing pMPX-6::t-inv in the absence of t-inv expression, naked pMPX-6::t-inv alone, and naked pMPX-6::t-inv with lipofectamine. Each of these events will be monitored using fluorescent microscopy and/or flow cytometry. From these results the specific targeting apparatus proteins will be tested. Using A-431 (display EGFR) and K-562 (no EGFR) cell lines, the pMPX-6::t-egf constructs will be tested. Using the same approaches as for the t-inv study, the level of transfection between A-431 and K-562 cell lines will be measured and compared to those achieved using lipofectamine. Similarly, the ability of TNF to stimulate gene transfer will be studied using L-929 cells. In all cases, the ability of these general and specific targeting protein constructs will be compared to standard transfection techniques. Upon positive results, these methodologies will be tested on difficult to transfect cell lines, e.g. adult cardiomyocytes. The basis of these results will create a foundation for which applications into in vivo gene transfer may occur.

#### 25      **EXAMPLE 20:            ADDITIONAL AND OPTIMIZED METHODS FOR GENETIC    EXPRESSION**

Expression in minicells may occur following purification of minicells and/or minicell protoplasts from parental cells and LPS constituents, respectively. However, for some applications it is preferred to co-express proteins of interest with minicell induction. For these approaches, one may use the protocol described in Example 13 for expression of the phoA constructs. Either of these approaches may be accomplished using one or more of the following expression constructs (Table 23) and/or optimized expression constructs (Table 25).

Expression plasmid pCGV1 contains a temperature sensitive lambda cI repressor (cI857) and both lambda PR and PL promoters (Guzman, C. A., et al. 1994. A novel Escherichia coli expression-export vector containing alkaline phosphatase as an insertional inactivation screening system. *Gene*. 148:171-172) with an atpE initiation region (Schauder, B., et al. 1987. Inducible expression vectors incorporating the Escherichia coli atpE translational initiation region. *Gene*. 52:279-283). Included in the design of the invention is the modification of this expression vector to best align the required Shine-Delgarno ribosomal binding site with cloning sites. In addition, the pCGVI expression vector was modified to incorporate a stem-loop structure at the 3-prime end of the transcript in order to provide a strong transcriptional stop sequence (Table 23).

Expression plasmid pCL478 contains a temperature sensitive lambda cI repressor (cI857) and both lambda PR and PL promoters (Love, C. A., et al. 1996. Stable high-copy bacteriophage promoter vectors for overproduction of proteins in Escherichia coli. *Gene*. 176:49-53). Included in the design of the invention is the modification of this expression vector to best align the required Shine-Delgarno ribosomal binding site with cloning sites. In addition, the pCL478 expression vector was modified to incorporate a stem-loop structure at the 3-prime end of the transcript in order to provide a strong transcriptional stop sequence (Table 23).

**TABLE 23. LAMBDA C1857 EXPRESSION VECTOR MODIFICATIONS**

New Plasmid	Parent plasmid	Region removed	Region added <sup>a</sup>	SEQ ID NO
pMPX-84	pCGV1	NdeI - BamHI	NdeI, SD - PstI, XbaI, KpnI, Stem-loop, BamHI	139
pMPX-85	pCGV1	NdeI - BamHI	NdeI, SD - SalI, XbaI, KpnI, Stem-loop, BamHI	140
pMPX-86	pCL478	BamHI - XhoI	BamHI, SD - PstI, XbaI, KpnI, Stem-loop, XhoI	141
pMPX-87	pCL478	BamHI - XhoI	BamHI, SD - SalI, XbaI, KpnI, Stem-loop, XhoI	142

a. "SD" refers to a Shine-Delgarno ribosome-binding sequence; "Stem-loop" refers to a stem-loop structure that functions as a transcriptional stop site.

**TABLE 24. OLIGONUCLEOTIDE PRIMER SEQUENCES FOR TABLE 23**

SEQ ID NO	Primer name	5' to 3' sequence
143	CGV1-1-SaII	TATGTAAGGAGGTTGTCGACCGGCTCAGTCTAGAGGTACCCGCCCTCA TCCGAAAGGGCGTATTG
144	CGV1-2-SaII	GATCCAATACGCCCTTTTCGGATGAGGGCGGGTACCTCTAGACTGAGCC GGTCGACAACCTCCTTACA
145	CGV1-1-PstI	TATGTAAGGAGGTTTCTGCAGCGGCTCAGTCTAGAGGTACCCGCCCTCA TCCGAAAGGGCGTATTG
146	CGV1-2-PstI	GATCCAATACGCCCTTTTCGGATGAGGGCGGGTACCTCTAGACTGAGCC GCTGCAGAACCTCCTTACA
147	CL478-1-SaII	GATCCTAAGGAGGTTGTCGACCGGCTCAGTCTAGAGGTACCCGCCCTC ATCCGAAAGGGCGTATTG
148	CL478-2-SaII	TCGAGAATACGCCCTTTTCGGATGAGGGCGGGTACCTCTAGACTGAGCC GGTCGACAACCTCCTTAG
149	CL478-1-PstI	GATCCTAAGGAGGTTTCTGCAGCGGCTCAGTCTAGAGGTACCCGCCCTC ATCCGAAAGGGCGTATTG
150	CL478-2-PstI	TCGAGAATACGCCCTTTTCGGATGAGGGCGGGTACCTCTAGACTGAGCC GCTGCAGAACCTCCTTAG

5 Oligonucleotides SEQ ID NOS.: 143 and 144 were annealed to each other to generate a DNA molecule with a 5' overhang at both ends. The overhangs are designed so that the DNA can be directly cloned into pCGVI cut with NdeI (5' overhang is TA) and BamHI (5' overhang is GATC). Insertion of the annealed DNA into pCGVI creates SEQ ID NO.: 139, pMPX-84.

10 Oligonucleotides SEQ ID NOS.: 145 and 146 were annealed to each other to generate a DNA molecule with a 5' overhang at both ends. The overhangs are designed so that the DNA can be directly cloned into pCGVI cut with NdeI (5' overhang is TA) and BamHI (5' overhang is GATC). Insertion of the annealed DNA into pCGVI creates SEQ ID NO.: 140, pMPX-85.

15 Oligonucleotides SEQ ID NOS.: 147 and 148 were annealed to each other to generate a DNA molecule with a 5' overhang at both ends. The overhangs are designed so that the DNA can be directly cloned into pCL478 cut with BamHI (5' overlap is GATC) and XhoI (overhang is TCGA). Insertion of the annealed DNA into pCL578 cut with BamHI and XhoI creates SEQ ID NO.: 141, pMPX-86.

20 Oligonucleotides SEQ ID NOS.: 149 and 150 were annealed to each other to generate a DNA molecule with a 5' overhang at both ends. The overhangs are designed so that the DNA can be directly cloned into pCL578 cut with BamHI (5' overlap is

The optimized expression constructs in Table 25 were created from SEQ ID NOS.: 6, 11, and 12 (see Table 13). Modifications were made to optimize the alignment of the SalI or PstI cloning sites with the Shine-Delgarno ribosome-binding site. In addition, stem-loop transcriptional termination sequences were added on the 3' end of the cloning region.

10

SEQ ID NO.:	Primer name	5' to 3' sequence
69	Rha-1	GCGAATTGAGATGACGCCACTGGC
156	Rha-SD	GCAGAACTCTCTGAATTCATTACGACC
71	Rha-1-HindIII	CGGCGAAAGCTTAATTAATCTTTCTGCGAATTGAGATGACGCCACTGGC
157	Rha-SD SalI KpnI	CCGCGGGTACCAATACGCCCTTTCGGATGAGGGC GCGGGGATCCTCTAGAGTCGACGTCGACAACTCTC CTGAATTTCAATTACGACC
158	Rha-SD KpnI KpnI	CCGCGGGTACCAATACGCCCTTTCGGATGAGGGC GCGGGGATCCTCTAGAGTCGACCTGCGAAGAACTCT CTGAATTTCAATTACGACC
81	Ara-1	CAAGCCGTCAATTGTCTGATTCTG
159	Ara-SD	CTGCAGGGCCTCTGCTAGCCCAAAAAACGGGTATGG
83	Ara-1-HindIII	GCGCCAAGCTTCAAGCCGTCAATTGTCTGATTCTG
160	Ara-SD SalI KpnI	CCGCGGGTACCAATACGCCCTTTCGGATGAGGGC GCGGGGATCCTCTAGAGTCGACGTCGACGGCCTCT CTGCTAGCCCAAAAAACGGGTATGG

SEQ ID NO.:	Primer name	5' to 3' sequence
161	Ara-SD PstI KpnI	CCGCGGGTACCAATACGCCCTTTCGGATGAGGGC GCGGGGATCCTCTAGAGTCGACCTGCAGGGCCTC CTGCTAGCCCAAAAAACGGGTATGG
77	Mel-1	CGTCTTTAGCCGGGAAACG
162	Mel-SD	CCTCCTGGCTTGCTTGAATAAATTCATCATGG
79	Mel-1-HindIII	GCCGCAAGCTTCGTCCTTAGCCGGGAAACG
163	Mel-SD-SalI KpnI	CCGCGGGTACCAATACGCCCTTTCGGATGAGGGC GCGGGGATCCTCTAGAGTCGACCCCTCTCTGGCT TGCTTGAATAACTTCATCATGGC

Oligonucleotides SEQ ID NOS.: 69, 156, 72, and 157 were used to amplify the rhaRS genes and their divergent control region from the E. coli chromosome and insertion of an optimized SalI-Shine-Delgarno ribosome-binding alignment and a stem-loop transcriptional termination sequence. Once amplified, this region was inserted into pUC18 using HindIII and KpnI to create pMPX67, SEQ ID NO.: 151.

Oligonucleotides SEQ ID NOS.: 69, 156, 72, and 158 were used to amplify the rhaRS genes and their divergent control region from the E. coli chromosome and insertion of an optimized PstI-Shine-Delgarno ribosome-binding alignment and a stem-loop transcriptional termination sequence. Once amplified, this region was inserted into pUC18 using HindIII and KpnI to create, pMPX-72, SEQ ID NO.: 152.

Oligonucleotides SEQ ID NOS.: 81, 159, 81, 160 were used to amplify the araC genes and their divergent control region from the E. coli chromosome and insertion of an optimized SalI-Shine-Delgarno ribosome-binding alignment and a stem-loop transcriptional termination sequence. Once amplified, this region was inserted into pUC18 using HindIII and KpnI to create, pMPX-66, SEQ ID NO.: 153.

Oligonucleotides SEQ ID NOS.: 81, 159, 81, 161 were used to amplify the araC genes and their divergent control region from the E. coli chromosome and insertion of an optimized PstI-Shine-Delgarno ribosome-binding alignment and a stem-loop transcriptional termination sequence. Once amplified, this region was inserted into pUC18 using HindIII and KpnI to create pMPX-71, SEQ ID NO.: 154.

WO 03/072014

PCT/US02/16877

Oligonucleotides SEQ ID NOS.: 77, 162, 79, 163 were used to amplify the melR genes and their divergent control region from the E. coli chromosome and insertion of an optimized SalI-Shine-Delgarno ribosome-binding alignment and a stem-loop transcriptional termination sequence. Once amplified, this region was

5 inserted into pUC18 using HindIII and KpnI to create, pMPX-68, SEQ ID NO.: 155.

#### EXAMPLE 21: OPTIMIZATION OF RAT NEUROTENSIN RECEPTOR (NTR) EXPRESSION

Expression of specific GPCR proteins in minicells may require chimeric domain

10 fusions to stabilize the expressed protein and/or direct the synthesized protein to the membrane. The NTR protein from rat was cloned into several chimeric combinations to assist in NTR expression and membrane association (Grishammer, R., et al. 1993. Expression of a rat neurotensin receptor in Escherichia coli. Biochem. J. 295:571-576; Tucker, J., and Grishammer, R. 1996. Purification of a rat neurotensin receptor expressed

15 in Escherichia coli. Biochem. J. 317:891-899). Methods for construction are shown the Tables below.

**TABLE 27. NEUROTENSIN RECEPTOR EXPRESSION FACILITATING CONSTRUCTS**

Protein <sup>a</sup>	Construct <sup>b</sup>	SEQ ID NO
MalE(L)	SalI-MalE (1-370)-Factor Xa-NTR homology	164
NTR	Factor Xa-NTR (43-424)-NotI-FLAG-KpnI	165
MalE(L)-NTR	SalI-MalE(1-370)-Factor Xa-NTR(43-424)-NotI-FLAG-KpnI	166
MalE(S)-NTR	SalI-MalE(1-28)-Factor Xa-NTR(43-424)-NotI-FLAG-KpnI	167
TrxA	NotI-TrxA(2-109)-NotI	168
MalE(L)-NTR-TrxA	SalI-MalE(1-370)-Factor Xa-NTR(43-424)-NotI-TrxA(2-109)-FLAG-KpnI	169
MalE(S)-NTR-TrxA	SalI-MalE(1-28)-Factor Xa-NTR(43-424)-NotI-TrxA(2-109)-FLAG-KpnI	170

- 20 a. (L) refers to MalE residues 1-370, and (S) refers to MalE residues 1-28.  
 b. All mature constructs were cloned into SalI and KpnI sites of SEQ ID NOS.: 140, 142, 151 and 153.

25

**TABLE 28. OLIGONUCLEOTIDE PRIMER SEQUENCES FOR TABLE 27**

SEQ ID NO.:	Primer name	5' to 3' sequence
171	MalE-1	GGTGCACGCATCCTCGCATTATCCGC
172	MalE-2	CGCACGGCATACCGAGAAAGCGGACATCTGCG
173	MalE-1-SalI	CCGCGGTCGACATGAAAATAAAACAGGTGCACGC ATCCTCGC
174	MalE-2-XaNTR	GCCGTGTCCGATTCCGAGGTGCGGCCTTCGATACGC ACGGCAT ACCAAGAAAGCGGGATGTTTCGGC
175	NTR-1	CCTCGGAATCCGACACGGCAGGGC
176	NTR-2	GTACAGGCTCTCCCGGTGGCGCTGG
177	NTR-1-Xa	CCGCGATCGAAGGCCGCACCTCGGAATCCGACACG GCAGGGCC
178	NTR-2-Flag	GGCGCGGTACCTTTGTATCATCGTCATCTTTATAATCT GCGGCCG GTACAGGCTCTCCCGGTGGCGCTGGTGG
179	NTR-2-Stop KpnI	GCGCGGTACCTTATTATTGTATCATCGTCATCTTTAT AATCTGC GGCCGCG
180	NTR-1-Xa Lead	CCGCATTAACGACGATGATGTTTTCCGCCTCGGCTC TCGCCAA ATCATCGAAGGCCGCACCTCGGAATCCGACACGGC
181	NTR-2-Lead2 SalI	CCGCGGTCGACATGAAAATAAAACAGGTGCACGC ATCCTCGC ATTATCCGCATTAAACGACGATGATGTTTTCCGCCTC GGC
182	TrxA-1	CCGCGAGCGATAAAATTATTCACCTGACTGACG
183	TrxA-2	GCCCGCCAGGTTAGCGTCGAGGAACCTTTCAACTG ACC
184	TrxA-1-NotI	GCGGCCGCAAGCGATAAAATTATTCACCTGACTGA CG
185	TrxA-2-NotI	GGCGCTGCGGCCGCATCATGATCTTTATAATCG CC

Oligonucleotides SEQ ID NOS.: 171, 172, 173 and 174 were used to amplify malE residues 1-370 from the E. coli chromosome to create SEQ ID NO.: 164. Using overlap PCR with the extended NTR homology, a chimeric translational fusion was made between MalE (1-370) and NTR residues 43-424 (SEQ ID NO.: 165) to create a SEQ ID NO.: 166. SEQ ID NO.: 166 was cloned into plasmids pMPX-85, pMPX-87, pMPX-66 and pMPX-67 (respectively, SEQ ID NOS.: 140, 142, 151 and 153) using SalI and KpnI.

Three-step PCR with oligonucleotides, SEQ ID NOS.: 175 and 176 as primers was used to amplify NTR residues 43-424 from rat brain cDNA. SEQ ID NOS.: 177 and 178 were then used with the NTR (43-424) template to add factor Xa and FLAG sequence.

WO 03/072014

PCT/US02/16877

Finally, SEQ ID NOS.: 177 and 179 were used to add a KpnI site to create SEQ ID NO.: 165. Using overlap PCR with malE(1-370) containing extended NTR homology, a chimeric translational fusion was made between NTR (43-424) and MalE (1-370) (SEQ ID NO.: 164) to create a SEQ ID NO.: 166. SEQ ID NO.: 166 was cloned into SEQ ID NOS.: 140, 142, 151 and 153 using SalI and KpnI.

Using three-step PCR oligonucleotides SEQ ID NOS.: 175 and 176 were first used to amplify NTR residues 43-424 from rat brain cDNA. SEQ ID NOS.: 178 and 180 were then used with the NTR (43-424) template to add factor Xa and FLAG sequence. Finally, SEQ ID NOS.: 179 and 181 were used to add KpnI to create SEQ ID NO.: 167. SEQ ID NO.: 167 was cloned into SEQ ID NOS.: 140, 142, 151 and 153 using SalI and KpnI.

Oligonucleotides SEQ ID NOS.: 182, 183, 184 and 185 were used to amplify TrxA residues 2-109 from the E. coli chromosome to create SEQ ID NO.: 168. Using NotI, TrxA residues 2-109 was cloned into SEQ ID NOS.: 166 and 167 to create SEQ ID NOS.: 169 and 170, respectively. SEQ ID NO.: 169 and 170 were cloned into SEQ ID NOS.: 140, 142, 151 and 153 using SalI and KpnI.

## EXAMPLE 22: METHODS FOR FUNCTIONAL GPCR ASSAY

Functional G-protein-coupled receptor (GPCR) binding assays in minicells requires expression of a GPCR of interest into the minicell membrane bilayer and cytoplasmic expression of the required G-protein. For these purposes, constructs were created to co-express both a GPCR and a G-protein. To regulate the ratio of GPCR to G-protein, transcriptional fusions were created. In these constructs, the GPCR and G-protein are co-transcribed as a bi-cistronic mRNA. To measure the GPCR-G-protein interaction in the intact minicell, each protein was created as a chimera with a transactivation domain. For these studies the N-terminal DNA-binding, activation domain of the ToxR protein from *V. cholerae* was fused to the C-terminus of both the GPCR and G-protein. Finally, to measure the interaction GPCR-G-protein interaction, the ToxR-activated ctx promoter region was cloned in front of lacZ. Dimerization of the ToxR DNA-binding region will bind and activate the ctx promoter. In this construct, heterodimerization of the GPCR and G-protein will promote dimerization of ToxR that will be monitored by LacZ expression. Details of these constructs are shown in Table 29.

TABLE 29. FUNCTIONAL HUMAN GPCR CONSTRUCTS

Protein <sup>a, b</sup>	Construct <sup>a, b</sup>	SEQ ID NO.:
$\beta 2AR$	Sall- $\beta 2AR$ -PstI, XhoI	186
GS1 $\alpha$	XhoI-GS1 $\alpha$ -XbaI	187
$\beta 2AR$ -GS1 $\alpha$ fusion	Sall- $\beta 2AR$ -PstI, XhoI-GS1 $\alpha$ -XbaI	188
$\beta 2AR$ -stop	Sall- $\beta 2AR$ -PstI-Stop-SD-XhoI	189
$\beta 2AR$ -stop-GS1 $\alpha$	Sall- $\beta 2AR$ -PstI-Stop-SD-XhoI-GS1 $\alpha$ -XbaI	190
ToxR	Clal-ToxR-XbaI	191
GS1 $\alpha$	XhoI-GS1 $\alpha$ -Clal	192
GS2 $\alpha$	XhoI-GS2 $\alpha$ -Clal	193
G $\alpha$ q	XhoI-G $\alpha$ q-Clal	194
G $\alpha$ i	XhoI-G $\alpha$ i-Clal	195
G $\alpha$ 12/13	XhoI-G $\alpha$ 12/13-Clal	196
GS1 $\alpha$ -ToxR	XhoI-GS1 $\alpha$ -Clal-ToxR-XbaI	197
GS2 $\alpha$ -ToxR	XhoI-GS2 $\alpha$ -Clal-ToxR-XbaI	198
G $\alpha$ q-ToxR	XhoI-G $\alpha$ q-Clal-ToxR-XbaI	199
G $\alpha$ i-ToxR	XhoI-G $\alpha$ i-Clal-ToxR-XbaI	200
G $\alpha$ 12/13-ToxR	XhoI-G $\alpha$ 12/13-Clal-ToxR-XbaI	201
ToxR	PstI-ToxR-XhoI	202
$\beta 2AR$	Sall- $\beta 2AR$ -PstI	203
$\beta 2AR$ -ToxR	Sall- $\beta 2AR$ -PstI-ToxR-Stop-SD-XhoI	204
$\beta 2AR$ -ToxR-stop-GS1 $\alpha$ -ToxR	Sall- $\beta 2AR$ -PstI-ToxR-Stop-SD-XhoI-GS1 $\alpha$ -Clal-ToxR-XbaI	205
Pctx	XbaI-Pctx-lacZ homology	206
lacZ	Pctx homology-lacZ-XbaI	207
Pctx::lacZ	XbaI-Pctx-lacZ-XbaI	208

- a. "SD" refers to the Shine-Delgarno ribosome-binding sequence and "ToxR" refers to the transactivation, DNA-binding domain of the ToxR protein (residues 5-141).
- b. All mature constructs were cloned into Sall and XbaI sites of SEQ ID NOS.: 140, 142, 151 and 153.

TABLE 30. OLIGONUCLEOTIDE PRIMER SEQUENCES FOR TABLE 29.

SEQ ID NO.:	Primer name	5' to 3' sequence
209	$\beta 2AR$ -1	GGGGCAACCCGGGAACGGCAGCGCC
210	$\beta 2AR$ -2	GCAGTGAGTCATTGTACTACAATTCTCC
211	$\beta 2AR$ -1-Sall	CGCGGTCGACATGGGGCAACCCGGGAACGGCAGCGCC
212	$\beta 2AR$ -2-Link-XhoI	GGCTCGAGCTGCAGGTTGGTGACCGCTCGGCCACGCTCTAGCAGTGAGTCATTGTACTACAATTCC
213	GS1 $\alpha$ -1	GGGCTGCCTCGGGAACAGTAAGACCGAGG
214	GS1 $\alpha$ -2	GAGCAGCTCGTACTGACGAAGGTGCATGC
215	GS1 $\alpha$ -1-XhoI	GGAGGCCCTCGAGATGGGCTGCCTCGGGAACAGTAAGACCGAGG

WO 03/072014

PCT/US02/16877

SEQ ID NO.:	Primer name	5' to 3' sequence
216	GS1 $\alpha$ -2-XbaI	CCTCTAGATTATTATCGATGAGCAGCTCGTACTGACGAAGGTGCATGC
217	GS1 $\alpha$ -2-Clal	CCATCGATGAGCAGCTCGTACTGACGAAGGTGCATGC
218	G $\alpha$ 12-1	CCGGGGTGGTGC GGACCTCAGCCGC
219	G $\alpha$ 12-2	CTGCAGCATGATGTCCTTCAGGTTCTCC
220	G $\alpha$ 12-1-XhoI	GCGGGCTCGAGATGTCCGGGGTGGTGGGACCCCTCAGC CGC
221	G $\alpha$ 12-2-Clal	GCGCCATCGATCTGCAGCATGATGTCCTTCAGGTTCTCC
222	G $\alpha$ q-1	GACTCTGGAGTCCATCATGGCGTCTCTGC
223	G $\alpha$ q-2	CCAGATTGTACTCCTTCAGGTTCAACTGG
224	G $\alpha$ q-1-XhoI	ATGACTCTGGAGTCCATCATGGCGTCTCTGC
225	G $\alpha$ q-2-Clal	GCGCCATCGATGACCAGATTGTACTCCTTCAGGTTCAACTGG
226	G $\alpha$ 1-1	GGGCTGCACCGTGAGCGCCGAGGACAAGG
227	G $\alpha$ 1-2	CCTTCAGGTTGTCTTGATGATGACATCGG
228	G $\alpha$ 1-1-XhoI	ATGGGCTGCACCGTGAGCGCCGAGGACAAGG
229	G $\alpha$ 1-2-Clal	GCGCCATCGATGAAGAGGCCGAGTCCCTTCAGGTTGTCTTGA TGATGACATCGG
230	GS2 $\alpha$ -1	GGGCTGCCTCGGGAACAGTAAGACCGAGG
231	GS2 $\alpha$ -2	GAGCAGCTCGTACTGACGAAGGTGCATGC
232	GS2 $\alpha$ -1-XhoI	ATGGGCTGCCTCGGGAACAGTAAGACCGAGG
233	GS2 $\alpha$ -2-Clal	GCGCCATCGATGAGCAGCTCGTACTGACGAAGGTGCATGC
234	$\beta$ 2AR-2-Link-Stop-XhoI	GGCTCGAGGGCCTCCTTGATTATTACTCGAGGGCCTCC TTGATTATTACTGCAGGTTGGTGACCGTCTGGCCACGC TCTAGCAGTGAGTCATTGTACTACAATTCC
235	$\beta$ 2AR-2-Link	CCCTGCAGGTTGGTGACCGTCTGGCCACGCTCTAGCAG TGAGTCATTGTACTACAATTCC
236	Tox (5-141)-1B	GGACACAACCTAAAAGAGATATCGATGAGTCATATTG G
237	Tox (5-141)-2	GAGATGTCATGAGCAGCTTCGTTTTCGCG
238	Tox (5-141)-1-Link	GCGTGCCAGACGGTCACCAACCTGCAGGGACACAAC TCAAAAAGAGATATCG
239	Tox (5-141)-2-XhoI	GCGGGATCCTCTAGATTATTAAGAGATGTCATGAGCAG CTTCGTTTTCGCG
240	Ctx-1	GGCTGTGGGTGAAGTGAACGGGGTTTACCG
241	Ctx-2	CTTTACCATATAATGCTCCCTTTGTTTAAACAG
242	Ctx-2-XbaI	CGCGGTCTAGAGGCTGTGGGTAGAAGTGAACGGGGT TTACCG
243	Ctx-2-LacZ	CGACGGCCAGTGAATCCGTAATCATGGTCTTTACCATA TAATGCTCCCTTTGTTTAAACAG
244	LacZ-1	CCATGATTACGGATTCACTGGCCGTGC

WO 03/072014

PCT/US02/16877

SEQ ID NO.:	Primer name	5' to 3' sequence
245	LacZ-2	CCAGACCAACTGGTAATGGTAGCGACC
246	LacZ-1-Ctx	GGTAAAGACCATGATTACGGATTCACTGGCCGTCG
247	LacZ-2-XbaI	GCGCCTCTAGAAATACGCCCTTTTCGGATGAGGGCGTT ATTATTTTIGACACCAGACCAACTGGTAATGGTAGCG ACC

Oligonucleotides SEQ ID NOS.: 209, 210, 211 and 212 were used to amplify human  $\beta$ 2AR from human cDNA to create SEQ ID NO.: 186. Using SalI and XhoI a translational fusion was made between  $\beta$ 2AR and human GS1 $\alpha$  (SEQ ID NO.: 187) to create a SEQ ID NO.: 188. SEQ ID NO.: 188 was cloned into SEQ ID NOS.: 140, 142, 151 and 153 using SalI and XbaI.

Oligonucleotides SEQ ID NOS.: 213, 214, 215 and 216 were used to amplify human GS1 $\alpha$  from human cDNA to create SEQ ID NO.: 187. Using XhoI and XbaI a translational fusion was made between GS1 $\alpha$  and human  $\beta$ 2AR (SEQ ID NO.: 186) create SEQ ID NO.: 188. SEQ ID NO.: 188 was cloned into SEQ ID NOS.: 140, 142, 151 and 153 using SalI and XbaI.

Oligonucleotides SEQ ID NOS.: 213, 214, 215 and 217 were used to amplify human GS1 $\alpha$  from human cDNA to create SEQ ID NO.: 192. Using XhoI and XbaI a translational fusion was made with ToxR residues 5-141 from *Vibrio cholerae* (SEQ ID NO.: 191) to create SEQ ID NO.: 197. To be used to create a transcriptional fusion with  $\beta$ 2AR-ToxR chimeras as shown in SEQ ID NO.: 205 and future GPCR-ToxR chimeras.

Oligonucleotides SEQ ID NOS.: 218, 219, 220 and 221 were used to amplify human G $\alpha$ 12/13 from human cDNA to create SEQ ID NO.: 196. Using XhoI and XbaI a translational fusion was made with ToxR residues 5-141 from *Vibrio cholerae* (SEQ ID NO.: 191) to create SEQ ID NO.: 201. To be used to create future transcriptional fusions with GPCR-ToxR chimeras as shown in SEQ ID NO.: 205.

Oligonucleotides SEQ ID NOS.: 222, 223, 224 and 225 were used to amplify human G $\alpha$ q from human cDNA to create SEQ ID NO.: 194. Using XhoI and XbaI a translational fusion was made with ToxR residues 5-141 from *Vibrio cholerae* (SEQ ID NO.: 191) to create SEQ ID NO.: 199. To be used to create future transcriptional fusions with GPCR-ToxR chimeras as shown in SEQ ID NO.: 205.

WO 03/072014

PCT/US02/16877

Oligonucleotides SEQ ID NOS.: 226, 227, 228 and 229 were used to amplify human  $\text{G}\alpha$  from human cDNA to create SEQ ID NO.: 195. Using XhoI and XbaI a translational fusion was made with ToxR residues 5-141 from *Vibrio cholerae* (SEQ ID NO.: 191) to create SEQ ID NO.: 200. To be used to create future transcriptional fusions with GPCR-  
5 ToxR chimeras as shown in SEQ ID NO.: 205.

Oligonucleotides SEQ ID NOS.: 230, 231, 232 and 233 were used to amplify human  $\text{GS}\alpha$  from human cDNA to create SEQ ID NO.: 193. Using XhoI and XbaI a translational fusion was made with ToxR residues 5-141 from *Vibrio cholerae* (SEQ ID NO.: 191) to create SEQ ID NO.: 198. To be used to create future transcriptional fusions with GPCR-  
10 ToxR chimeras as shown in SEQ ID NO.: 205.

Oligonucleotides SEQ ID NOS.: 209, 210, 211 and 234 were used to amplify human  $\beta$ 2AR from human cDNA to create SEQ ID NO.: 189. Using SalI and XhoI a transcriptional fusion was made between  $\beta$ 2AR and human  $\text{GS}\alpha$  (SEQ ID NO.: 187) to create a SEQ ID NO.: 190. SEQ ID NO.: 190 was cloned into SEQ ID NOS.: 140, 142, 151 and 153 using  
15 SalI and XbaI.

Oligonucleotides SEQ ID NOS.: 236, 237, 238 and 239 were used to amplify bases coinciding with ToxR residues 5-141 from *Vibrio Cholerae* to create SEQ ID NO.: 202. Using PstI and XhoI a translational fusion was made between ToxR and human  $\beta$ 2AR (SEQ ID NO.: 203) to create SEQ ID NO.: 204.

Oligonucleotides SEQ ID NOS.: 209, 210, 211 and 235 were used to amplify human  $\beta$ 2AR from human cDNA to create SEQ ID NO.: 203. Using SalI and PstI a translational fusion was made between  $\beta$ 2AR and ToxR (SEQ ID NO.: 202) to create SEQ ID NO.: 204.  
20

Using oligonucleotides SEQ ID NOS.: 197 and 204 transcriptional fusions were created between the  $\beta$ 2AR-ToxR translational fusion (SEQ ID NO.: 204) and the  $\text{GS}\alpha$ -ToxR  
25 translational fusion (SEQ ID NO.: 197) to create SEQ ID NO.: 205.

Oligonucleotides SEQ ID NOS.: 240, 241, 242 and 243 were used to amplify the ctx promoter region (Ctx) from *Vibrio cholerae* to create SEQ ID NO.: 206. Combining this PCR product in combination with the SEQ ID NO.: 207 PCR product and amplifying in the presence of SEQ ID NOS.: 242, 247, SEQ ID NO.: 208 was created. Using XbaI, the SEQ

Oligonucleotides SEQ ID NOS.: 244, 245, 246 and 247 were used to amplify the lacZ from E. coli to create SEQ ID NO.: 207. Combining this PCR product in combination with the SEQ ID NO.: 206 PCR product and amplifying in the presence of SEQ ID NOS.: 242 and 247, SEQ ID NO.: 208 was created. Using XbaI, the 208 reporter construct was subsequently cloned into pACYC184 for co-transformation with the GPCR-G-protein fusions constructs above.

To produce membrane proteins efficiently in minicells it may be necessary to create chimeric fusions with the membrane protein of interest. In this Example, various regions of the MalE protein have been cloned into a modular expression system designed to create chimeric fusions with direct difficult to target membrane proteins to produce leader domains that will direct the proteins to the cytoplasmic membrane ( Miller, K., W., et al. 1998. Production of active chimeric pediocin AcH in *Escherichia coli* in the absence of processing and secretion genes from the *Pediococcus* pap operon. *Appl. Environ. Microbiol.* 64:14-20). Similarly, a modified version of the TrxA protein has also been cloned into this modular expression system to create chimeric fusions with proteins that are difficult to maintain in a soluble conformation (LaVallie, E. R., et al. 1993. A thioredoxin gene fusion expression system that circumvents inclusion body formation in the *E. coli* cytoplasm. *Biotechnology* (N. Y.) 11:187-193). Table 31 describes each of these modular constructs.

25      **TABLE 31.    MODULAR MEMBRANE-TARGETING  
AND SOLUBILIZATION EXPRESSION CONSTRUCTS**

348

Protein <sup>a</sup>	Construct <sup>a</sup>	SEQ ID NO
MalE (1-28)-TrxA (2-109, del 103-107)	Nsil-MalE(1-28)-Factor Xa-PstI, SalI, XbaI-TrxA (2-109 del 103-107)-FLAG, NheI	251
MalE (1-370, del 354-364)-TrxA (2-109, del 103-107)	Nsil-MalE(1-370, del 354-364)-Factor Xa-PstI, SalI, XbaI-TrxA (2-109 del 103-107)-FLAG, NheI	252

a. The term "del" refers to a deletion in which amino acid residues following the term "del" are removed from the sequence.

**TABLE 32. OLIGONUCLEOTIDE PRIMER SEQUENCES FOR TABLE 31.**

5

SEQ ID NO.:	Primer name	5' to 3' sequence
253	MalE-1-Nsil	CGCGGATGCATATGAAAAATAAAACAGGTGCACGCAT CCTCGCATTATCCGCATTAAACGACGATGATGTTTCCG CCTCGGCTCTCGCC
254	MalE-2-middle	CGTCGACCGAGGCCTGCAGGCGGGCTTCGATGATTTT GGCGAG AGCCGAGGCGGAAAAACATCATCTCGTCG
255	MalE-3s-NheI	CGAAGCCCGCCTGCAGGCCTCGGTCGACGCCGAATCT AGAGATTATAAAGATGACGATGACAAATAATAAGCTA GCGGCGC
256	MalE-4-NheI	GCGCCGCTAGCTTATTATTGTTCATCG
257	MalE-1a	GGTGACGCACTCCTCGCATTATCCGC
258	MalE-2a	GGCGTTTCCATGGTGGCGCAATACGTGG
259	MalE-1-Nsil	CGCGGATGCATATGAAAAATAAAACAGGTGCACGCAT CCTC GCATTATCCGC
260	MalE-2-NheI	CCGAGGCCTGCAGGCGGGCTTCGATACGCACGGCATA CCAG AAAGCGGACTGGCGTTTTCATGGTGGCGGCAATAC GTGG
261	MalE-3L-NheI	GCGCCGCTAGCTTATTATTGTTCATCGTCATCTTTATA ATCTC TAGATTGCGGCTCGACCGAGGCCTGCAGGCGGGCTTC GATA CGC
262	TrxA-1a	CCTGACTGACGACAGTTTTGACACGG
263	TrxA-2a	CCTTTAGACAGTGACCCCACTTTGGTTGCCCG

WO 03/072014

PCT/US02/16877

SEQ ID NO.:	Primer name	5' to 3' sequence
264	TrxA-1a-PstI	CGCGGCTGCAGGCCTCGGTCGACGCCGAATCTAGAAG CGAT AAAATTATTACCTGACTGACGACAGITTTTGACACGG
265	TrxA-2-NheI	GCGCCGCTAGCTTATTATTGTCATCGTCATCTTTATA ATCCG CCAGGTTCTCTTCAACTGACCTTTAGACAGTGCACCC ACTTT GGTTGCCGC

Oligonucleotides SEQ ID NOS.: 253, 254, 255 and 256 overlap with each other to form a scaffold template to PCR amplify malE (1-28) to create a SEQ ID NO.: 248.

Following PCR amplification, SEQ ID NO.: 248 was digested with NsiI and NheI and cloned into SEQ ID NOS.: 152, 154, 139 and 141 digested with PstI and XbaI. The resultant products create SEQ ID NOS.: 266, 267, 268 and 269, respectively, that lose both the 5-prime PstI and 3-prime XbaI restriction site and retain the PstI, SalI, and XbaI restriction sites between MalE (1-28) and the FLAG sequence. Insertion of a protein in alignment with these sites results in a chimeric protein containing amino-terminal MalE (1-28) and carboxy-terminal FLAG.

Oligonucleotides SEQ ID NOS.: 257, 258, 259 and 260 were used to amplify malE (1-370 with a deletion removing residues 354-364) to create SEQ ID NO.: 249. Following PCR amplification, SEQ ID NO.: 249 was digested with NsiI and NheI and cloned into SEQ ID NOS.: 152, 154, 139 and 141 digested with PstI and XbaI. The resultant products create SEQ ID NOS.: 270, 271, 272 and 273, respectively, that lose both the 5-prime PstI and 3-prime XbaI restriction site and retain the PstI, SalI, and XbaI restriction sites between MalE (1-370, del 354-364) and the FLAG sequence. Insertion of a protein in alignment with these sites results in a chimeric protein containing amino-terminal MalE (1-370, del 354-364) and carboxy-terminal FLAG.

Oligonucleotides SEQ ID NOS.: 262, 263, 264 and 265 were used to amplify trxA (2-109 with a deletion removing residues 103-107) to create SEQ ID NO.: 250. Following PCR amplification, SEQ ID NO.: 250 was digested with PstI and NheI and cloned into SEQ ID NOS.: 152, 154, 139 and 141 digested with PstI and XbaI. to create SEQ ID NOS.: 274,

WO 03/072014

PCT/US02/16877

275, 276 and 277, respectively. Using these restriction digestion combinations results in loss of the XbaI SEQ ID NO.: 249 insertion site.

The resultant products create SEQ ID NOS.: 274, 275, 276 and 277, respectively, that lose the 3-prime XbaI restriction site and retain the PstI, SalI, and XbaI restriction sites  
5 on the 3-prime end of the TrxA (1-109, del 103-107) sequence. Insertion of a protein in alignment with these sites results in a chimeric protein containing Carboxy-terminal TrxA (1-109, del 103-107)-FLAG.

SEQ ID NO.: 248 was digested with NsiI and XbaI and cloned into SEQ ID NOS.: 274, 275, 276 and 277 that were digested with PstI and XbaI. The resultant products create  
10 SEQ ID NOS.: 278, 279, 280 and 281, respectively, that lose the 5 prime PstI restriction site and retain the PstI, SalI, and XbaI restriction sites between MalE (1-28) and TrxA (1-109, del 103-107). Insertion of a protein in alignment with these sites results in a chimeric protein containing amino-terminal MalE (1-28) and carboxy-terminal TrxA (1-109, del 103-107)-FLAG.

15 SEQ ID NO.: 249 was digested with NsiI and XbaI and cloned into SEQ ID NOS.: 274, 275, 276 and 277 that were digested with PstI and XbaI. The resultant products create SEQ ID NOS.: 282, 283, 284 and 285, respectively, that lose the 5 prime PstI restriction site and retain the PstI, SalI, and XbaI restriction sites between MalE (1-370, del 354-364) and TrxA (1-109, del 103-107). Insertion of a protein in alignment with these sites results in a  
20 chimeric protein containing amino-terminal MalE (1-370, del 354-364) and carboxy-terminal TrxA (1-109, del 103-107)-FLAG.

#### EXAMPLE 24: POROPLAST<sup>TM</sup> FORMATION

Minicells are used to prepare Poroplasts in order to increase the accessibility of a  
25 membrane protein component and/or domain to the outside environment. Membrane proteins in the inner membrane are accessible for ligand binding and/or other interactions in poroplasts, due to the absence of an outer membrane. The removal of the outer membrane from E. coli whole cells and minicells to produce poroplasts was carried out using modifications of previously described protoplast and analysis protocols (Birdsell et al.,  
30 Production and Ultrastructure of Lysozyme and Ethylenediaminetetraacetate-Lysozyme Spheroplasts of Escherichia coli, J. Bacteriol. 93:427-437, 1967; Weiss et al., Protoplast

WO 03/072014

PCT/US02/16877

Formation in *Escherichia coli*, J. Bacteriol. 128:668-670, 1976; Matsuyama, S-I., et al. SecD is involved in the release of translocated secretory proteins from the cytoplasmic membrane of *Escherichia coli*. 12:265-270, 1993).

In brief, cells were grown to late-log phase and pelleted at room temperature.

- 5 Minicells were also isolated from cultures in late-log phase. The pellet was washed twice with 50 mM Tris, pH 8.0. Following the second wash,  $1 \times 10^9$  cells were resuspended in 1 ml 50 mM Tris (pH 8.0) that contained 8 % sucrose and 2 mM EDTA. Cell/EDTA/sucrose mixtures were incubated at 37°C for 10 min, centrifuged, decanted, and poroplasted cells were resuspended in 50 mM Tris, pH 8.0 with 8 % sucrose. Incubation with anti-LPS-coated  
10 magnetic beads, as described in Example 14, is used to enrich for poroplasts that lack LPS. Following incubation with the resuspended protoplasted cells, the anti-LPS magnetic beads were removed from suspension with a magnet.

- To examine the range of molecular sizes that can pass through the cell wall, an IgG molecule was tested for its ability to pass the intact cell wall. Binding of an antibody to the  
15 ToxR-PhoA chimera expressed on the inner membrane minicell poroplasts was measured. Briefly, minicell poroplasts with and without inner membrane-bound ToxR-PhoA were incubated at 37°C with anti-PhoA antibody in reaction buffer (50 mM Tris, pH 8.0, 8 % sucrose, 1 % BSA, and 0.01 % Tween-20). Following incubation, poroplasts were centrifuged, washed 3 times with reaction buffer, and resuspended in 50 mM Tris, pH 8.0  
20 with 8 % sucrose. Following resuspension, bound proteins from  $5 \times 10^7$  minicells or minicell poroplasts were separated using denaturing SDS-PAGE, transferred to nitrocellulose, and developed using with both anti-PhoA antibody and secondary antibody against both heavy and light chains of anti-PhoA IgG (Table 33).

TABLE 33: ANTI-PHOA ACCESSIBILITY TO POROPLAST  
INNER MEMBRANE-BOUND TOXR-PHOA

25

EDTA (mM)	0	2	0	2
Lysozyme (mg/ml)	0	0	5	5
	Poroplasts (ng antibody bound)		Protoplasts (ng antibody bound)	

WO 03/072014

PCT/US02/16877

Minicells ToxR-PhoA	ND <sup>a</sup>	0.6	ND <sup>a</sup>	12.8
Minicells only	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>

a. Non-detectable

These results demonstrate that the cell wall present on poroplasts is penetrable by an IgG molecule and that an IgG molecule is capable of passing the intact cell wall and binding to an inner membrane protein. From this data it appears that poroplast operate at ~ 10% the efficiency of protoplasts by allowing 0.6 ng of IgG to bind inner membrane-bound ToxR-PhoA compared to 12.8 ng. However, given the large size of IgG (~ 150,000 Daltons) it is expected that molecules having a smaller molecular weight will efficiently access inner membrane proteins in poroplasts.

**EXAMPLE 25: PRODUCTION OF NEUROTENSIN RECEPTOR (NTR).**

To demonstrate expression of NTR in isolated minicells, MalE(L)-NTR (SEQ ID NO.: 166 was cloned into pMPX-67 (SEQ ID NO.: 151). Following minicell isolation,  $1.5 \times 10^9$  minicells were induced with 1 mM Rhamnose for 2 hour at 37°C. Following induction, the protein produced was visualized via Western analysis using an anti-MalE antibody following separation on an SDS-PAGE. The results are shown in Figure 2.

These data demonstrates that MalE(L)-NTR is induced 87-fold by addition of 1 mM rhamnose to the minicell induction mixture. Cross-reactive proteins are host MalE and non-specific binding by Goat-anti-mouse HRP secondary antibody.

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The contents of the articles, patents, and patent applications, and all other documents and electronically available information mentioned or cited herein, are hereby incorporated by reference in their entirety to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference. Applicants reserve the right to physically incorporate into this application any and all materials and information from any such articles, patents, patent applications, or other documents.

WO 03/072014

PCT/US02/16877

The inventions illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including," containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein

5 have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional

10 features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form

15 part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

Other embodiments are within the following claims. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art

20 will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

## CLAIMS

1. A minicell comprising a membrane protein selected from the group consisting of a eukaryotic membrane protein, an archaeobacterial membrane protein and an organellar membrane protein.
- 5 2. The minicell of claim 1, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.
3. The minicell of claim 1, wherein said minicell comprises a biologically active compound.
4. The minicell of claim 1, wherein said minicell comprises a expression construct,  
10 wherein said first expression construct comprises expression sequences operably linked to an ORF that encodes a protein.
5. The minicell of claim 4, wherein said ORF encodes said membrane protein.
6. The minicell of claim 4, wherein said expression sequences that are operably linked to an ORF are inducible and/or repressible.
- 15 7. The minicell of claim 4, wherein said minicell comprises a second expression construct, wherein said second expression construct comprises expression sequences operably linked to a gene.
8. The minicell of claim 7, wherein said expression sequences that are operably linked to a gene are inducible and/or repressible.
- 20 9. The minicell of claim 7, wherein the gene product of said gene regulates the expression of the ORF that encodes said protein.
10. The minicell of claim 7, wherein the gene product of said gene is a nucleic acid.
11. The minicell of claim 7, wherein the gene product of said gene is a polypeptide.
12. The minicell of claim 11, wherein said polypeptide is a membrane protein, a soluble protein or a secreted protein.
- 25 13. The minicell of claim 12, wherein said membrane protein is a membrane fusion protein, said membrane fusion protein comprising a first polypeptide, said first polypeptide comprising at least one transmembrane domain or at least one membrane anchoring domain; and a second polypeptide.

WO 03/072014

PCT/US02/16877

14. A minicell comprising a membrane fusion protein, said fusion protein comprising a first polypeptide, said first polypeptide comprising at least one transmembrane domain or at least one membrane anchoring domain; and a second polypeptide, wherein said second polypeptide is not derived from a eubacterial protein and is neither a His tag nor a glutathione-S-transferase polypeptide.
- 5
15. The minicell of claim 14, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.
16. The minicell of claim 14, wherein said minicell comprises a biologically active compound.
- 10
17. A minicell comprising a membrane conjugate, wherein said membrane conjugate comprises a membrane protein chemically linked to a conjugated compound.
18. The minicell of claim 17, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.
19. The minicell of claim 17, wherein said minicell comprises a biologically active compound.
- 15
20. The minicell of claim 17, wherein said conjugated compound is selected from the group consisting of a nucleic acid, a polypeptide, a lipid and a small molecule.
21. A method for making minicells, comprising
- (a) culturing a minicell-producing parent cell, wherein said parent cell comprises an expression construct, wherein said expression construct comprises a gene operably linked to expression sequences that are inducible and/or repressible, and wherein induction or repression of said gene causes or enhances the production of minicells; and
- 20
- (b) separating said minicells from said parent cell, thereby generating a composition comprising minicells,
- 25
- wherein an inducer or repressor is present within said parent cells during one or more steps and/or between two or more steps of said method.
22. The method of claim 21, further comprising
- (c) purifying said minicells from said composition.

WO 03/072014

PCT/US02/16877

23. The method of claim 21, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.
24. The method of claim 21, wherein said gene expresses a gene product that is a factor that is involved in or modulates DNA replication, cellular division, cellular  
5 partitioning, septation, transcription, translation, or protein folding.
25. The method of claim 21, wherein said minicells are separated from said parent cells by a process selected from the group consisting of centrifugation, ultracentrifugation, density gradation, immunoaffinity and immunoprecipitation.
26. The method of claim 22, wherein said minicell is a poroplast, said method further  
10 comprising
- (d) treating said minicells with an agent, or incubating said minicells under a set of conditions, that degrades the outer membrane of said minicell.
27. The method of claim 26, wherein said outer membrane is degraded by treatment with an agent selected from the group consisting of EDTA, EGTA, lactic acid, citric acid,  
15 gluconic acid, tartaric acid, polyethyleneimine, polycationic peptides, cationic leukocyte peptides, aminoglycosides, aminoglycosides, protamine, insect cecropins, reptilian magainins, polymers of basic amino acids, polymyxin B, chloroform, nitrilotriacetic acid and sodium hexametaphosphate and/or by exposure to conditions selected from the group consisting of osmotic shock and insonation.
28. The method of claim 26, further comprising removing one or more contaminants from said composition.
29. The method of claim 28, wherein said contaminant is LPS or peptidoglycan.
30. The method of claim 29, wherein said LPS is removed by contacting said composition to an agent that binds or degrades LPS.
31. The method of claim 21, wherein said minicell-producing parent cell comprises a  
25 mutation in a gene required for lipopolysaccharide synthesis.
32. The method of claim 22, wherein said minicell is a spheroplast, said method further comprising
- (d) treating said minicells with an agent, or incubating said minicells under a set  
30 of conditions, that disrupts or degrades the outer membrane; and

WO 03/072014

PCT/US02/16877

- (e) treating said minicells with an agent, or incubating said minicells under a set of conditions, that disrupts or degrades the cell wall.
33. The method of claim 32, wherein said agent that disrupts or degrades the cell wall is a lysozyme, and said set of conditions that disrupts or degrades the cell wall is incubation in a hypertonic solution.
34. The method of claim 22, wherein said minicell is a protoplast, said method further comprising
- (d) treating said minicells with an agent, or incubating said minicells under a set of conditions, that disrupt or degrade the outer membrane;
- (e) treating said minicells with an agent, or incubating said minicells under a set of conditions, that disrupts or degrades the cell wall, in order to generate a composition that comprises protoplasts; and
- (f) purifying protoplasts from said composition.
35. The method of claim 22, further comprising preparing a denuded minicell from said minicell.
36. The method of claim 22, further comprising covalently or non-covalently linking one or more components of said minicell to a conjugated moiety.
37. A method of preparing a L-form minicell comprising:
- (a) culturing an L-form eubacterium, wherein said eubacterium comprises one or more of the following:
- (i) an expression element that comprises a gene operably linked to expression sequences that are inducible and/or repressible, wherein induction or repression of said gene regulates the copy number of an episomal expression construct;
- (ii) a mutation in an endogenous gene, wherein said mutation regulates the copy number of an episomal expression construct.
- (iii) an expression element that comprises a gene operably linked to expression sequences that are inducible and/or repressible, wherein induction or repression of said gene causes or enhances the production of minicells; and

WO 03/072014

PCT/US02/16877

- (iv) a mutation in an endogenous gene, wherein said mutation causes or enhances minicell production.
- (b) culturing said L-form minicell-producing parent cell in media under conditions wherein minicells are produced; and
- 5 (c) separating said minicells from said parent cell, thereby generating a composition comprising L-form minicells,
- wherein an inducer or repressor is present within said minicells during one or more steps and/or between two or more steps of said method.
38. The method of claim 37, further comprising
- 10 (d) purifying said L-form minicells from said composition.
39. A method of producing a protein, comprising:
- (a) transforming a minicell-producing parent cell with an expression element that comprises expression sequences operably linked to a nucleic acid having an ORF that encodes said protein;
- 15 (b) culturing said minicell-producing parent cell under conditions wherein minicells are produced; and
- (c) purifying minicells from said parent cell,
- (d) purifying said protein from said minicells.
- wherein said ORF is expressed during step (b), between steps (b) and (c), and during step (c).
- 20 40. The method of claim 39, wherein said expression elements segregate into said minicells, and said ORF is expressed between steps (c) and (d).
41. The method of claim 39, wherein said protein is a membrane protein.
42. The method of claim 39, wherein said protein is a soluble protein contained within said minicells, further comprising:
- 25 (e) at least partially lysing said minicells.
43. The method of claim 39, wherein said protein is a secreted protein, wherein said method further comprises

- (e) collecting a composition in which said minicells are suspended or with which said minicells are in contact.
44. The method of claim 39, wherein the expression sequences to which said ORF is operably linked are inducible, wherein said method further comprises adding an inducing agent between steps (a) and (b); during step (b); and between steps (b) and (c).
45. The method of claim 39, wherein the expression sequences to which said ORF is operably linked are inducible, wherein said expression elements segregate into said minicells, said method further comprises adding an inducing agent after step (c).
46. The method of claim 39, further comprising:
- (e) preparing poroplasts from said minicells,
- wherein said ORF is expressed during step (b); between steps (b) and (c); during step (c); between step (c) and step (d) when said expression elements segregate into said minicells; and/or after step (d) when said expression elements segregate into said minicells.
47. The method of claim 46, further comprising:
- (f) purifying said protein from said poroplasts.
48. The method of claim 39, further comprising
- (e) preparing spheroplasts from said minicells,
- wherein said ORF is expressed during step (b), between steps (b) and (c), during step (c), between steps (c) and (d) and/or after step (d).
49. The method of claim 48, further comprising:
- (f) purifying said protein from said spheroplasts.
50. The method of claim 39, further comprising
- (e) preparing protoplasts from said minicells,
- wherein said ORF is expressed during step (b), between steps (b) and (c), during step (c), between steps (c) and (d) and/or after step (d).
51. The method of claim 50, further comprising:
- (f) purifying said protein from said protoplasts.

WO 03/072014

PCT/US02/16877

52. The method of claim 39, further comprising
- (e) preparing membrane preparations from said minicells,
- wherein said ORF is expressed during step (b), between steps (b) and (c), during step (c), between steps (c) and (d) and/or after step (d).
- 5 53. The method of claim 48, further comprising:
- (f) purifying said protein from said membrane preparations.
54. The method of claim 39, wherein said minicell-producing parent cell is an L-form bacterium.
55. A method of producing a protein, comprising:
- 10 (a) transforming a minicell with an expression element that comprises expression sequences operably linked to a nucleic acid having an ORF that encodes said protein; and
- (b) incubating said minicells under conditions wherein said ORF is expressed.
56. The method of claim 55, further comprising:
- 15 (c) purifying said protein from said minicells.
57. The method of claim 55, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.
58. A method of producing a protein, comprising:
- (a) transforming a minicell-producing parent cell with an expression element that
- 20 comprises expression sequences operably linked to a nucleic acid having an ORF that encodes a fusion protein comprising said protein and a polypeptide, wherein a protease-sensitive amino acid sequence is positioned between said protein and said polypeptide;
- (b) culturing said minicell-producing parent cell under conditions wherein
- 25 minicells are produced;
- (c) purifying minicells from said parent cell, wherein said ORF is expressed during step (b); between steps (b) and (c); and/or after step (c) when said expression elements segregate into said minicells; and

WO 03/072014

PCT/US02/16877

- (d) treating said minicells with a protease that cleaves said sensitive amino acid sequence, thereby separating said protein from said polypeptide.
59. A poroplast, said poroplast comprising a vesicle, bonded by a membrane, wherein said membrane is an eubacterial inner membrane, wherein said vesicle is surrounded by a eubacterial cell wall, and wherein said eubacterial inner membrane is accessible to a compound in solution with said poroplast.
60. The poroplast of claim 59, wherein said poroplast is a cellular poroplast.
61. The poroplast of claim 59, wherein said compound has a molecular weight of at least 1 kD.
62. The poroplast of claim 59, wherein said poroplast comprises an exogenous nucleic acid.
63. The poroplast of claim 62, wherein said exogenous nucleic acid is an expression construct.
64. The poroplast of claim 63, wherein said expression construct comprises an ORF that encodes an exogenous protein, wherein said ORF is operably linked to expression sequences.
65. The poroplast of claim 64, wherein said poroplast comprises an exogenous protein.
66. The poroplast of claim 59, wherein said poroplast comprises an exogenous protein.
67. The poroplast of claim 66, wherein said exogenous protein is a fusion protein, a soluble protein or a secreted protein.
68. The poroplast of claim 66, wherein said exogenous protein is a membrane protein.
69. The poroplast of claim 68, wherein said membrane protein is accessible to compounds in solution with said poroplast.
70. The poroplast of claim 68, wherein said membrane protein is selected from the group consisting of a eukaryotic membrane protein, an archaeobacterial membrane protein, and an organellar membrane protein.
71. The poroplast of claim 68, wherein said membrane protein is a fusion protein, said fusion protein comprising a first polypeptide, said first polypeptide comprising at least one transmembrane domain or at least one membrane anchoring domain; and a second polypeptide, wherein said second polypeptide is displayed by said poroplast.

WO 03/072014

PCT/US02/16877

72. The poroplast of claim 71, wherein said second polypeptide is displayed on the external side of said eubacterial inner membrane.
73. The poroplast of claim 59, wherein said poroplast comprises a membrane component that is chemically linked to a conjugated compound.
- 5 74. The poroplast of claim 64, wherein said expression construct comprises one or more DNA fragments from a genome or cDNA.
75. The poroplast of claim 64, wherein said exogenous protein has a primary amino acid sequence that is predicted from in silico translation of a nucleic acid sequence.
76. A method of making poroplasts or cellular poroplasts, comprising treating eubacterial  
10 minicells or cells with an agent, or incubating said minicells or cells under a set of conditions, that degrades the outer membrane of said minicells or cells.
77. The method of claim 76, further comprising purifying said poroplasts or cellular poroplasts in order to remove contaminants.
78. The method of claim 76, further comprising placing said poroplasts in a hypertonic  
15 solution, wherein 90% or more of said cells or minicells used to prepare said poroplasts would lyse in said solution under the same conditions.
79. A solid support comprising a minicell.
80. The solid support of claim 79, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.
- 20 81. The solid support of claim 79, wherein said solid support is a dipstick.
82. The solid support of claim 79, wherein said solid support is a bead.
83. The solid support of claim 79, wherein said solid support is a microtiter multiwell plate.
84. The solid support of claim 79, wherein said minicell comprises a detectable  
25 compound.
85. The solid support of claim 84, wherein said detectable compound is a fluorescent compound.
86. The solid support of claim 79, wherein said minicell displays a membrane component.

WO 03/072014

PCT/US02/16877

87. The solid support of claim 86, wherein said membrane component is selected from the group consisting of (i) a eukaryotic membrane protein, (ii) an archaeobacterial membrane protein, (iii) an organellar membrane protein, (iv) a fusion protein comprising at least one transmembrane domain or at least one membrane anchoring domain, and (v) a membrane conjugate comprising a membrane component chemically linked to a conjugated compound.
88. The solid support of claim 86, wherein said membrane component is a receptor.
89. The solid support of claim 87, wherein said solid support further comprises a co-receptor.
90. The solid support of claim 79, wherein said minicell displays a binding moiety.
91. A solid support comprising a minicell, wherein said minicell displays a fusion protein, said fusion protein comprising a first polypeptide that comprises at least one transmembrane domain or at least one membrane anchoring domain, and a second polypeptide.
92. The solid support of claim 91, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.
93. The solid support of claim 91, wherein said second polypeptide comprises a binding moiety.
94. The solid support of claim 91, wherein said second polypeptide comprises an enzyme moiety.
95. A solid support comprising a minicell, wherein said minicell comprises a membrane conjugate comprising a membrane component chemically linked to a conjugated compound.
96. The solid support of claim 95, wherein said conjugated compound is a spacer.
97. The solid support of claim 96, wherein said spacer is covalently linked to said solid support.
98. The solid support of claim 95, wherein said conjugated compound is covalently linked to said solid support.
99. A minicell comprising a biologically active compound, wherein said minicell displays a binding moiety, wherein said binding moiety is part of a fusion protein comprising a first polypeptide that comprises at least one transmembrane domain or at least one

membrane anchoring domain and a second polypeptide that comprises a binding moiety, and said minicell is a poroplast, spheroplast or protoplast.

100. A eubacterial minicell comprising a biologically active compound, wherein said minicell displays a binding moiety, wherein said binding moiety is selected from the group consisting of (a) a eukaryotic membrane protein; (b) an archaeobacterial membrane protein; (c) an organellar membrane protein; and (d) a fusion protein, said fusion protein comprising a first polypeptide, said first polypeptide comprising at least one transmembrane domain or at least one membrane anchoring domain; and a second polypeptide, wherein said second polypeptide is not derived from a eubacterial protein and is neither a His tag nor a glutathione-S-transferase polypeptide, and wherein said polypeptide comprises a binding moiety.
101. The minicell of claim 99, wherein said binding moiety is selected from the group consisting of an antibody, an antibody derivative, a receptor and an active site of a non-catalytic derivative of an enzyme.
102. The minicell of claim 99, wherein said binding moiety is a single-chain antibody.
103. The minicell of claim 99, wherein said binding moiety is directed to a ligand selected from the group consisting of an epitope displayed on a pathogen, an epitope displayed on an infected cell and an epitope displayed on a hyperproliferative cell.
104. The minicell of claim 99, wherein said biologically active compound is selected from the group consisting of a radioisotope, a polypeptide, a nucleic acid and a small molecule.
105. The minicell of claim 99, further comprising a first and second nucleic acid, wherein said first nucleic acid comprises eukaryotic expression sequences operably linked to a first ORF, and a second nucleic acid, wherein said second nucleic acid comprises eubacterial expression sequences operably linked to a second ORF.
106. The minicell of claim 105, wherein one of said ORFs encodes a protein that comprises said binding moiety.
107. The minicell of claim 105, wherein said eubacterial expression sequences are induced and/or derepressed when said binding moiety is in contact with a target cell.
108. The minicell of claim 105, wherein said eukaryotic expression sequences are induced and/or derepressed when said nucleic acid is in the cytoplasm of a eukaryotic cell.

WO 03/072014

PCT/US02/16877

109. The minicell of claim 105, wherein the protein encoded by said first ORF comprises eukaryotic secretion sequences and/or the protein encoded by said second ORF comprises eubacterial secretion sequences.
- 5 110. A method of associating a radioactive compound with a cell, wherein said cell displays a ligand specifically recognized by a binding moiety, comprising contacting said cell with a minicell that comprises said radioactive compound and displays said binding moiety.
111. The method of claim 110, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.
- 10 112. The method of claim 110, wherein the amount of radiation emitted by said radioactive isotope is sufficient to be detectable.
113. The method of claim 110, wherein the amount of radiation emitted by said radioactive isotope is sufficient to be cytotoxic.
114. The method of claim 110, wherein said ligand displayed by said cell is selected from the group consisting of an epitope displayed on a pathogen, an epitope displayed on an infected cell and an epitope displayed on a hyperproliferative cell.
- 15 115. The method of claim 110, wherein said binding moiety is selected from the group consisting of an antibody, an antibody derivative, a channel protein protein and a receptor.
- 20 116. The method of claim 110, wherein said binding moiety is a single-chain antibody.
117. The method of claim 110, wherein said binding moiety is selected from the group consisting of an aptamer and a small molecule.
118. A method of delivering a biologically active compound to a cell, wherein said cell displays a ligand specifically recognized by a binding moiety, comprising contacting said cell with a minicell that displays said binding moiety, wherein said minicell comprises said biologically active compound, and wherein the contents of said minicell are delivered into said cell from a minicell bound to said cell.
- 25 119. The method of claim 118, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.

WO 03/072014

PCT/US02/16877

120. The method of claim 118, wherein said biologically active compound is selected from the group consisting of a nucleic acid, a lipid, a polypeptide, a radioactive compound, an ion and a small molecule.
121. The method of claim 118, wherein the membrane of said minicell comprises a system for transferring a molecule from the interior of a minicell into the cytoplasm of said cell.
122. The method of claim 121, wherein said system for transferring a molecule from the interior of a minicell into the cytoplasm of said cell is a Type III secretion system.
123. The method of claim 118, wherein said minicell further comprises a first and second nucleic acid, wherein said first nucleic acid comprises eukaryotic expression sequences operably linked to a first ORF, and a second nucleic acid, wherein said second nucleic acid comprises eubacterial expression sequences operably linked to a second ORF.
124. The method of claim 123, wherein one of said ORFs encodes a protein that comprises said binding moiety.
125. The method of claim 123, wherein said eubacterial expression sequences are induced and/or derepressed when said binding moiety is in contact with a target cell.
126. The method of claim 123, wherein said eukaryotic expression sequences are induced and/or derepressed when said nucleic acid is in the cytoplasm of a eukaryotic cell.
127. The method of claim 123, wherein the protein encoded by said first ORF comprises eukaryotic secretion sequences and/or the protein encoded by said second ORF comprises eubacterial secretion sequences.
128. A minicell displaying a synthetic linking moiety, wherein said synthetic linking moiety is covalently or non-covalently attached to a membrane component of said minicell.
129. The minicell of claim 128, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.
130. A sterically stabilized minicell comprising a displayed moiety that has a longer half-life in vivo than a wild-type minicell, wherein said displayed moiety is a hydrophilic polymer that comprises a PEG moiety, a carboxylic group of a polyalkylene glycol or PEG stearate.

WO 03/072014

PCT/US02/16877

131. A minicell having a membrane comprising an exogenous lipid, wherein a minicell comprising said exogenous lipid has a longer half-life in vivo than a minicell lacking said exogenous lipid, and wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.
- 5 132. The minicell of claim 131, wherein said exogenous lipid is a derivitized lipid.
133. The minicell of claim 132, wherein said derivitized lipid is selected from the group consisting of phosphatidylethanolamine derivitized with PEG, DSPE-PEG, PEG stearate; PEG-derivitized phospholipids, and PEG ceramides is DSPE-PEG.
134. The minicell of claim 131, wherein said exogenous lipid is not present in a wild-type membrane, or is present in a different proportion than is found in minicells comprising a wild-type membrane,
- 10 135. The minicell of claim 134, wherein said exogenous lipid is selected from the group consisting of ganglioside, sphingomyelin, monosialoganglioside GM1, galactocerebroside sulfate, 1,2-sn-dimyristoylphosphatidylcholine, phosphatidylinositol and cardiolipin.
- 15 136. The minicell of claim 128, wherein said linking moiety is non-covalently attached to said minicell.
137. The minicell of claim 136, wherein one of said linking moiety and said membrane component comprises biotin, and the other comprises avidin or streptavidin.
- 20 138. The minicell of claim 128, wherein said synthetic linking moiety is a cross-linker.
139. The minicell of claim 128, wherein said cross-linker is a bifunctional cross-linker.
140. A method of transferring a membrane protein from a minicell membrane to a biological membrane comprising contacting a minicell to said biological membrane, wherein said minicell membrane comprises said membrane protein, and allowing said minicell and said biological membrane to remain in contact for a period of time sufficient for said transfer to occur.
- 25 141. The method of claim 140, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.
142. The method of claim 140, wherein biological membrane is a cytoplasmic membrane or an organellar membrane.
- 30

WO 03/072014

PCT/US02/16877

143. The method of claim 140, wherein said biological membrane is a membrane selected from the group consisting of a membrane of a pathogen, a membrane of an infected cell and a membrane of a hyperproliferative cell.
144. The method of claim 140, wherein said biological membrane is the cytoplasmic  
5 membrane of a recipient cell.
145. The method of claim 144, wherein said recipient cell is selected from the group consisting of a cultured cell and a cell within an organism.
146. The method of claim 140, wherein biological membrane is present on a cell that has been removed from an animal, said contacting occurs in vitro, after which said cell is  
10 returned to said organism.
147. The method of claim 144, wherein said membrane protein is an enzyme.
148. The method of claim 147, wherein said membrane protein having enzymatic activity is selected from the group consisting of a cytochrome P450 and a fusion protein, said fusion protein comprising a first polypeptide, said first polypeptide comprising at  
15 least one polypeptide, wherein said second polypeptide has enzymatic activity.
149. The method of claim 140, wherein said membrane protein is a membrane fusion protein, said membrane fusion protein comprising a first polypeptide, said first polypeptide comprising at least one transmembrane domain or at least one membrane anchoring domain; and a second polypeptide.
- 20 150. The method of claim 149, wherein said second polypeptide is a biologically active polypeptide.
151. The method of claim 140, wherein said minicell displays a binding moiety.
152. A minicell that comprises an expression construct comprising an ORF encoding a membrane protein operably linked to expression sequences, wherein said expression  
25 sequences are induced and/or derepressed when said minicell is in contact with a target cell.
153. The minicell of claim 152, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.
154. The minicell of claim 152, wherein biological membrane is a cytoplasmic membrane  
30 or an organellar membrane.

155. The minicell of claim 152, wherein said biological membrane is a membrane selected from the group consisting of a membrane of a pathogen, a membrane of an infected cell and a membrane of a hyperproliferative cell.
156. The minicell of claim 152, wherein said minicell displays a binding moiety.
- 5 157. The minicell of claim 156, wherein said binding moiety is selected from the group consisting of an antibody, an antibody derivative, an aptamer and a small molecule.
158. The minicell of claim 152, wherein said membrane protein is a membrane fusion protein, said membrane fusion protein comprising a first polypeptide, said first polypeptide comprising at least one transmembrane domain or at least one membrane anchoring domain; and a second polypeptide.
- 10 159. The minicell of claim 152, wherein said membrane protein having enzymatic activity is selected from the group consisting of a cytochrome P450 and a fusion protein, said fusion protein comprising a first polypeptide, said first polypeptide comprising at least one polypeptide, wherein said second polypeptide has enzymatic activity.
- 15 160. A pharmaceutical composition comprising a minicell, wherein said minicell displays a membrane protein, wherein said membrane protein is selected from the group consisting of a eukaryotic membrane protein, an archaeobacterial membrane protein and an organellar membrane protein.
161. The pharmaceutical composition of claim 160, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.
- 20 162. The pharmaceutical composition of claim 160, wherein said membrane protein is selected from the group consisting of a receptor, a channel protein, a cellular adhesion factor and an integrin.
- 25 163. The pharmaceutical formulation of claim 162, wherein said pharmaceutical formulation further comprises an adjuvant.
164. The pharmaceutical formulation of claim 162, wherein said membrane protein comprises a polypeptide epitope displayed by a hyperproliferative cell.
- 30 165. The pharmaceutical formulation of claim 162, wherein said membrane protein comprises an epitope displayed by a eukaryotic pathogen, an archaeobacterial pathogen, a virus or an infected cell.

WO 03/072014

PCT/US02/16877

166. A pharmaceutical composition comprising a minicell, wherein said minicell displays a membrane protein that is a fusion protein, said fusion protein comprising (i) a first polypeptide, said first polypeptide comprising at least one transmembrane domain or at least one membrane anchoring domain; and (ii) a second polypeptide, wherein said second polypeptide is not derived from a eubacterial protein.
167. The pharmaceutical composition of claim 166, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.
168. The pharmaceutical formulation of claim 167, wherein said pharmaceutical formulation further comprises an adjuvant.
169. The pharmaceutical formulation of claim 167, wherein said second polypeptide comprises a polypeptide epitope displayed by a hyperproliferative cell.
170. The pharmaceutical formulation of claim 169, wherein said membrane protein comprises an epitope displayed by a eukaryotic pathogen, an archaeobacterial pathogen, a virus or an infected cell.
171. A pharmaceutical composition comprising a minicell, wherein said minicell displays a membrane conjugate, wherein said membrane conjugate comprises a membrane component chemically linked to a conjugated compound.
172. The pharmaceutical composition of claim 171, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.
173. The pharmaceutical composition of claim 171, wherein said membrane protein is selected from the group consisting of a receptor, a channel protein, a cellular adhesion factor and an integrin.
174. The pharmaceutical composition of claim 171, wherein said pharmaceutical further comprises an adjuvant.
175. The pharmaceutical composition of claim 171, wherein said membrane component is a polypeptide comprising at least one transmembrane domain or at least one membrane anchoring domain, or a lipid that is part of a membrane.

WO 03/072014

PCT/US02/16877

176. The pharmaceutical composition of claim 171, wherein said conjugated compound is a polypeptide, and the chemical linkage between said membrane compound and said conjugated compound is not a peptide bond.
177. The pharmaceutical composition of claim 171, wherein said conjugated compound is a nucleic acid.
178. The pharmaceutical composition of claim 171, wherein said conjugated compound is an organic compound.
179. The pharmaceutical composition of claim 176, wherein said organic compound is selected from the group consisting of a narcotic, a toxin, a venom, a sphingolipid and a soluble protein.
180. A method of making a pharmaceutical composition comprising a minicell, wherein said minicell displays a membrane protein, wherein said membrane protein is selected from the group consisting of a eukaryotic membrane protein, an archaeobacterial membrane protein and an organellar membrane protein.
181. The method of claim 1, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.
182. The method of claim 180, wherein said method further comprises adding an adjuvant to said pharmaceutical formulation.
183. The method of claim 180, wherein said method further comprises desiccating said formulation.
184. The method of claim 183, wherein said method further comprises adding a suspension buffer to said formulation.
185. The method of claim 180, wherein said method further comprises making a chemical modification of said membrane protein.
186. The method of claim 185, wherein said chemical modification is selected from the group consisting of glycosylation, deglycosylation, phosphorylation, dephosphorylation and proteolysis.
187. A method of making a pharmaceutical composition comprising a minicell, wherein said minicell displays a membrane protein that is a fusion protein, said fusion protein comprising (i) a first polypeptide, said first polypeptide comprising at least one transmembrane domain or at least one membrane anchoring domain; and (ii) a second

188. The method of claim 187, wherein said method further comprises adding an adjuvant to said pharmaceutical formulation.

189. The method of claim 187, wherein said method further comprises desiccating said pharmaceutical formulation.

190. The method of claim 189 wherein said method further comprises adding a suspension buffer to said pharmaceutical formulation.

191. The method of claim 187, wherein said method further comprises making a chemical modification of said membrane protein.

192. The method of claim 191, wherein said chemical modification is selected from the group consisting of glycosylation, deglycosylation, phosphorylation, dephosphorylation and proteolysis.

193. A method of making a pharmaceutical formulation comprising a minicell, wherein said minicell displays a membrane conjugate, wherein said membrane conjugate comprises a membrane component chemically linked to a conjugated compound.

194. The method of claim 193, wherein said method further comprises adding an adjuvant to said pharmaceutical formulation.

195. The method of claim 193, wherein said membrane component is a polypeptide comprising at least one transmembrane domain or at least one membrane anchoring domain, or a lipid that is part of a membrane.

196. The method of claim 193, wherein said conjugated compound is a polypeptide, and the chemical linkage between said membrane compound and said conjugated compound is not a peptide bond.

197. The method of claim 193, wherein said conjugated compound is a nucleic acid.

198. The method of claim 193, wherein said conjugated compound is an organic compound.

199. The method of claim 186, wherein said organic compound is selected from the group consisting of a narcotic, a toxin, a venom, and a sphingolipid.

- 374

- 375

- 376

238. A method of identifying an agent that specifically blocks the binding of a target compound to its ligand, comprising contacting the device of claim 236 with a library of compounds, and detecting a signal from said device, wherein said signal changes as a function of the binding of an agent to said target compound.
- 5 239. A method of making an antibody that specifically binds a protein domain, wherein said domain is in its native conformation, wherein said domain is contained within a protein displayed on a minicell, comprising contacting said minicell with a cell, wherein said cell is competent for producing antibodies to an antigen contacted with said cell, in order to generate an immunogenic response in which said cell produces said antibody.
- 10 240. The method of claim 239, wherein said minicell is a eubacterial minicell, a poroplast, a spheroplast or a protoplast.
241. The method of claim 239, wherein said protein displayed on a minicell is a membrane protein.
- 15 242. The method of claim 241, wherein said membrane protein is a receptor or a channel protein.
243. The method of claim 239, wherein said domain is found within the second polypeptide of a membrane fusion protein, wherein said membrane fusion protein comprises a first polypeptide, wherein said first polypeptide comprises at least one transmembrane domain or at least one membrane anchoring domain.
- 20 244. The method of claim 239, wherein said contacting occurs in vivo.
245. The method of claim 244, wherein said antibody is a polyclonal antibody or a monoclonal antibody.
246. The method of claim 244, wherein said contacting occurs in an animal that comprises an adjuvant.
- 25 247. The method of making an antibody derivative that specifically binds a protein domain, wherein said domain is in its native conformation, wherein said domain is displayed on a minicell, comprising contacting said minicell with a protein library, and identifying an antibody derivative from said protein library that specifically binds said protein domain.
- 30

WO 03/072014

PCT/US02/16877

248. The method of claim 247, wherein said protein library is selected from the group consisting of a phage display library, a phagemid display library, and a ribosomal display library.
249. The method of claim 247 wherein said antibody derivative is a single-chain antibody.
- 5 250. A method of making an antibody or antibody derivative that specifically binds an epitope, wherein said epitope is selected from the group consisting of (i) an epitope composed of amino acids found within a membrane protein, (ii) an epitope present in an interface between a membrane protein and a membrane component, (iii) an epitope present in an interface between a membrane protein and one or more other proteins  
10 and (iv) an epitope in a fusion protein, said fusion protein comprising a first polypeptide, said first polypeptide comprising at least one transmembrane domain or at least one membrane anchoring domain, and a second polypeptide, said second polypeptide comprising said epitope; comprising contacting a minicell displaying said epitope with a protein library, or to a cell, wherein said cell is competent for  
15 producing antibodies to an antigen contacted with said cell, in order to generate an immunogenic response in which said cell produces said antibody.
251. The method of claim 250, wherein said minicell is a eubacterial minicell, a poroplast, a spheroplast or a protoplast.
252. The method of claim 250, wherein said cell is contacted in vivo.
- 20 253. The method of claim 252, wherein said antibody is a polyclonal antibody.
254. The method of claim 252, wherein said antibody is a monoclonal antibody.
255. The method of claim 250, wherein said protein library is contacted in vitro.
256. The method of claim 255, wherein said protein library is selected from the group consisting of a phage display library, a phagemid display library, and a ribosomal  
25 display library.
257. The method of claim 256, wherein said antibody derivative is a single-chain antibody.
258. A method of determining the rate of transfer of nucleic acid from a minicell to a cell, comprising
  - (a) contacting said cell to said minicell, wherein said minicell comprises said  
30 nucleic acid, for a set period of time;
  - (b) separating minicells from said cells;

WO 03/072014

PCT/US02/16877

(c) measuring the amount of nucleic acid in said cells,

wherein the amount of nucleic acid in said cells over said set period of time is the rate of transfer of a nucleic acid from a minicell.

259. A method of determining the amount of a nucleic acid transferred to a cell from a minicell, comprising

(a) contacting said cell to said minicell, wherein said minicell comprises an expression element having eukaryotic expression sequences operably linked to an ORF encoding a detectable polypeptide, wherein said minicell displays a binding moiety, and wherein said binding moiety binds an epitope of said cell; and

(b) detecting a signal from said detectable polypeptide,

wherein a change in said signal corresponds to an increase in the amount of a nucleic acid transferred to a cell.

260. The method of claim 258, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.

261. The method of claim 258, wherein said cell is a eukaryotic cell.

262. The method of claim 258, wherein said binding moiety is an antibody or antibody derivative.

263. The method of claim 258, wherein said binding moiety is a single-chain antibody.

264. The method of claim 258, wherein said binding moiety is an aptamer.

265. The method of claim 258, wherein said binding moiety is an organic compound.

266. The method of claim 258, wherein said detectable polypeptide is a fluorescent polypeptide.

267. A method of detecting the expression of an expression element in a cell, comprising

(a) contacting said cell to a minicell, wherein said minicell comprises an expression element having cellular expression sequences operably linked to an ORF encoding a detectable polypeptide, wherein said minicell displays a binding moiety, and wherein said binding moiety binds an epitope of said cell;

- (b) incubating said cell and said minicell for a period of time effective for transfer of nucleic acid from said minicell to said cell; and
- (c) detecting a signal from said detectable polypeptide,
- wherein an increase in said signal corresponds to an increase in the expression of said expression element.
268. The method of claim 267, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.
269. The method of claim 267, wherein said cell is a eukaryotic cell and said expression sequences are eukaryotic expression sequences.
270. The method of claim 269, wherein said eukaryotic cell is a mammalian cell.
271. The method of claim 267, wherein said binding moiety is an antibody or antibody derivative.
272. The method of claim 267, wherein said binding moiety is a single-chain antibody.
273. The method of claim 267, wherein said binding moiety is an aptamer.
274. The method of claim 267, wherein said binding moiety is an organic compound.
275. The method of claim 267, wherein said detectable polypeptide is a fluorescent polypeptide.
276. A method for detecting the transfer of a fusion protein from the cytosol to an organelle of a eukaryotic cell, comprising
- (a) contacting said cell to a minicell, wherein
- (i) said minicell comprises an expression element having eukaryotic expression sequences operably linked to an ORF encoding a fusion protein, wherein said fusion protein comprises a first polypeptide that comprises organellar delivery sequences, and a second polypeptide that comprises a detectable polypeptide; and
- (ii) said minicell displays a binding moiety that binds an epitope of said cell, or an epitope of an organelle;

- 381

- 382

- (ii) said archaeobacterial membrane protein, (iii) said organellar membrane protein; and (iv) said fusion protein.
293. The method of claim 290, wherein said nucleic acid comprises an expression construct comprising expression sequences operably linked to an ORF, wherein said ORF encodes a therapeutic polypeptide.
294. The method of claim 293, wherein said expression sequences are inducible and/or derepressible.
295. The method of claim 294, wherein said expression sequences are induced or derepressed when the binding moiety displayed by said minicell binds its target compound.
296. The method of claim 294, wherein said expression sequences are induced or derepressed by a transactivation or transrepression event.
297. The method of claim 292, wherein said nucleic acid comprises an expression construct comprising expression sequences operably linked to an ORF, wherein said ORF encodes a polypeptide having an amino acid sequence that facilitates cellular transfer of a biologically active compound contained within or displayed by said minicell.
298. A minicell comprising a nucleic acid, wherein said nucleic acid comprises eukaryotic expression sequences and eubacterial expression sequences, each of which is independently operably linked to an ORF.
299. The minicell of claim 298, wherein said minicell is selected from the group consisting of a eubacterial minicell, a protoplast, a spheroplast and a protoplast.
300. The minicell of claim 298, wherein said minicell displays a binding moiety.
301. The minicell of claim 300, wherein said eubacterial expression sequences are induced and/or derepressed when said binding moiety is in contact with a target cell.
302. The minicell of claim 300, wherein said eukaryotic expression sequences are induced and/or derepressed when said nucleic acid is in the cytoplasm of a eukaryotic cell.
303. The minicell of claim 301, wherein the protein encoded by said ORF comprises eubacterial or eukaryotic secretion sequences.
304. A minicell comprising a first and second nucleic acid, wherein said first nucleic acid comprises eukaryotic expression sequences operably linked to a first ORF, and a

WO 03/072014

PCT/US02/16877

second nucleic acid, wherein said second nucleic acid comprises eubacterial expression sequences operably linked to a second ORF.

305. The minicell of claim 304, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.

5 306. The minicell of claim 304, wherein said minicell displays a binding moiety.

307. The minicell of claim 306, wherein said eubacterial expression sequences are induced and/or derepressed when said binding moiety is in contact with a target cell.

308. The minicell of claim 306, wherein said eukaryotic expression sequences are induced and/or derepressed when said nucleic acid is in the cytoplasm of a eukaryotic cell.

10 309. The minicell of claim 304, wherein the protein encoded by said first ORF comprises eukaryotic secretion sequences and/or the protein encoded by said second ORF comprises eubacterial secretion sequences.

310. A method of introducing into and expressing a nucleic acid in an organism, comprising contacting a minicell to a cell of said organism, wherein said minicell  
15 comprises said nucleic acid.

311. The method of claim 310, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.

312. The method of claim 310, wherein said minicell displays a binding moiety.

20 313. The method of claim 310, wherein said nucleic acid comprises a eukaryotic expression construct, wherein said eukaryotic expression construct comprises eukaryotic expression sequences operably linked to an ORF.

314. The method of claim 310, wherein said ORF encodes a protein selected from the group consisting of a membrane protein, a soluble protein and a protein comprising eukaryotic secretion signal sequences.

25 315. The method of claim 310, wherein said nucleic acid comprises a eubacterial expression construct, wherein said eubacterial expression construct comprises eubacterial expression sequences operably linked to an ORF.

316. The method of claim 315, wherein said minicell displays a binding moiety, wherein said eubacterial expression sequences are induced and/or derepressed when said  
30 binding moiety is in contact with a target cell.

WO 03/072014

PCT/US02/16877

317. The method of claim 316, wherein the protein encoded by said ORF comprises eubacterial secretion sequences.
318. A minicell comprising a crystal of a membrane protein.
319. The minicell of claim 318, wherein said minicell is a eubacterial minicell, a poroplast, a spheroplast or a protoplast.
320. The minicell of claim 318, wherein said membrane protein is a receptor.
321. The minicell of claim 320, wherein said receptor is a G-protein coupled receptor.
322. The minicell of claim 318, wherein said crystal is displayed.
323. The minicell of claim 318, wherein said membrane protein is a fusion protein, said fusion protein comprising a first polypeptide, said first polypeptide comprising at least one transmembrane domain or at least one membrane anchoring domain, and a second polypeptide.
324. The minicell of claim 323, wherein said crystal is a crystal of said second polypeptide.
325. The minicell of claim 323, wherein said crystal is displayed.
326. A method of determining the three-dimensional structure of a membrane protein, comprising preparing a crystal of said membrane protein in a minicell, and determining the three-dimensional structure of said crystal.
327. A method for identifying ligand-interacting atoms in a defined three-dimensional structure of a target protein, comprising (a) preparing one or more variant proteins of a target protein having a known or predicted three-dimensional structure, wherein said target protein binds a preselected ligand; (b) expressing and displaying a variant protein in a minicell; and (c) determining if a minicell displaying said variant protein binds said preselected ligand with increased or decreased affinity as compared to the binding of said preselected ligand to said target protein.
328. The method of claim 327, wherein said ligand is a protein that forms a multimer with said target protein, and said ligand interacting atoms are atoms in said defined three-dimensional structure are atoms that are involved in protein-protein interactions.
329. The method of claim 327, wherein said ligand is a compound that induces a conformational change in said target protein, and said defined three-dimensional structure is the site of said conformational change.

WO 03/072014

PCT/US02/16877

330. The method of claim 327, adopted to a method, said method for identifying ligands of a target protein, further comprising identifying the chemical differences in said variant proteins as compared to said target protein.
- 5 331. The method of claim 330, further comprising mapping said chemical differences onto said defined three-dimensional structure, and correlating the effect of said chemical differences on said defined three-dimensional structure.
332. The method of claim 331, wherein said target protein is a wild-type protein.
333. A minicell library, comprising two or more minicells, wherein each minicell comprises a different exogenous protein.
- 10 334. The minicell library of claim 333, wherein said minicell is a eubacterial minicell, a poroplast, a spheroplast or a protoplast.
335. The minicell library of claim 333, wherein said exogenous protein is a displayed protein.
- 15 336. The minicell library of claim 333, wherein said exogenous protein is a membrane protein.
337. The minicell library of claim 336, wherein said membrane protein is a receptor.
338. The minicell library of claim 333, wherein said protein is a soluble protein that is contained within or secreted from said minicell.
- 20 339. The minicell library of claim 333, wherein minicells within said library comprise an expression element that comprises expression sequences operably linked to a nucleic acid having an ORF that encodes said exogenous protein.
340. The minicell library of claim 339, wherein said nucleic acid has been mutagenized.
- 25 341. The minicell library of claim 339, wherein an active site of said exogenous protein has a known or predicted three-dimensional structure, and said a portion of said ORF encoding said active site has been mutagenized.
342. The minicell library of claim 333, wherein each of said minicells comprises an exogenous protein that is a variant of a protein having a known or predicted three-dimensional structure.
- 30 343. A minicell library, comprising two or more minicells, wherein each minicell comprises a different fusion protein, each of said fusion protein comprising a first

polypeptide that is a constant polypeptide, wherein said constant polypeptide comprises at least one transmembrane domain or at least one membrane anchoring domain, and a second polypeptide, wherein said second polypeptide is a variable amino acid sequence that is different in each fusion proteins.

- 5     344. The minicell library of claim 343, wherein minicells within said library comprise an expression element that comprises expression sequences operably linked to a nucleic acid having an ORF that encodes said fusion protein.
345. The minicell library of claim 344, wherein said second polypeptide of said fusion protein is encoded by a nucleic acid that has been cloned.
- 10    346. The minicell library of claim 344, wherein each of said second polypeptide of each of said fusion proteins comprises a variant of an amino acid sequence from a protein having a known or predicted three-dimensional structure.
347. A minicell library, comprising two or more minicells, wherein each minicell comprises a constant protein that is present in each minicell and a variable protein that differs from minicell to minicell.
- 15    348. The minicell library of claim 347, wherein one of said constant and variable proteins is a receptor, and the other of said constant and variable proteins is a co-receptor.
349. The minicell library of claim 347, wherein each of said constant and variable proteins is different from each other and is a factor in a signal transduction pathway.
- 20    350. The minicell library of claim 347, wherein one of said constant and variable proteins is a G-protein, and the other of said constant and variable proteins is a G-protein coupled receptor.
351. The minicell library of claim 347, wherein one of said constant and variable proteins comprises a first transrepression domain, and the other of said constant and variable
- 25    352. The minicell library of claim 347, wherein one of said constant and variable proteins comprises a first transactivation domain, and the other of said constant and variable
- 30    353. The minicell library of claim 347, wherein one of said constant and variable proteins comprises a second transactivation domain, wherein said transactivation domains stimulate expression of a reporter gene when said constant and variable proteins associate with each other.

353. A method of identifying a nucleic acid that encodes a protein that binds to or chemically alters a preselected ligand, comprising:

- (a) separately contacting said ligand with individual members of a minicell library, wherein minicells in said library comprise expression elements, wherein said expression elements comprise DNA inserts, wherein an ORF in said DNA insert is operably linked to expression sequences, in order to generate a series of reaction mixes, each reaction mix comprising a different member of said minicell library;
- (b) incubating said reaction mixes, thereby allowing a protein that binds to or chemically alters said preselected ligand to bind or chemically alter said preselected ligand;
- (c) detecting a change in a signal from reaction mixes in which said ligand has been bound or chemically altered;
- (d) preparing DNA from reaction mixes in which said ligand has been bound or chemically altered;

wherein said DNA is a nucleic acid that encodes a protein that binds to or chemically alters said preselected ligand.

354. The method of claim 353, wherein said minicell is a eubacterial minicell, a poroplast, a spheroplast or a protoplast.

355. The method of claim 353, wherein said preselected ligand is a biologically active compound.

356. The method of claim 353, wherein said preselected ligand is a therapeutic drug.

357. The method of claim 353, wherein a protein that binds or chemically alters said preselected ligand is a target protein for compounds that are therapeutic for a disease that is treated by administering said drug to an organism in need thereof.

358. The method of claim 353, wherein said preselected ligand is detectably labeled, said minicell comprises a detectable compound, and/or a chemically altered derivative of said protein is detectably labeled.

359. A method of determining the amino acid sequence of a protein that binds or chemically alters a preselected ligand, comprising:

WO 03/072014

PCT/US02/16877

- (a) contacting said ligand with a minicell library, wherein minicells in said library comprise expression elements, wherein said expression elements comprise DNA inserts, wherein an ORF in said DNA insert is operably linked to expression sequences;
- 5 (b) incubating said mixture of ligand and minicells, under conditions which allow complexes comprising ligands and minicells to form and/or chemical reactions to occur;
- (c) isolating or identifying said complexes from said ligand and said mixture of ligand and minicells;
- 10 (d) preparing DNA from an expression element found in one or more of said complexes, or in a minicell thereof;
- (e) determining the nucleotide sequence of said ORF in said DNA; and
- (f) generating an amino sequence by in silico translation, wherein said amino acid sequence is or is derived from a protein that binds or chemically alters a preselected ligand.
- 15 360. The method of claim 359, wherein said minicell is a eubacterial minicell, a poroplast, a spheroplast or a protoplast.
361. The method of claim 359, wherein said DNA is prepared by isolating DNA from said complexes, or in a minicell thereof.
- 20 362. The method of claim 359, wherein said DNA is prepared by amplifying DNA from said complexes, or in a minicell thereof.
363. The method of claim 359, wherein said protein is a fusion protein.
364. The method of claim 359, wherein said protein is a membrane or a soluble protein.
365. The method of claim 364, wherein said protein comprises secretion sequences.
- 25 366. The method of claim 359, wherein said preselected ligand is a biologically active compound.
367. The method of claim 359, wherein said preselected ligand is a therapeutic drug.
368. The method of claim 359, wherein said preselected ligand is a therapeutic drug, and said protein that binds said preselected ligand is a target protein for compounds that

WO 03/072014

PCT/US02/16877

are therapeutic for a disease that is treated by administering said drug to an organism in need thereof.

369. A method of identifying a nucleic acid that encodes a protein that inhibits or blocks an agent from binding to or chemically altering a preselected ligand, comprising:

- 5 (a) separately contacting said ligand with individual members of a minicell library, wherein minicells in said library comprise expression elements, wherein said expression elements comprise DNA inserts, wherein an ORF in said DNA insert is operably linked to expression sequences, in order to generate a series of reaction mixes, each reaction mix comprising a different member of said minicell library;
- 10 (b) incubating said reaction mixes, thereby allowing a protein that binds to or chemically alters said preselected ligand to bind or chemically alter said preselected ligand;
- (c) detecting a change in a signal from reaction mixes in which said ligand has been bound or chemically altered;
- 15 (d) preparing DNA from reaction mixes in which said change in signal ligand has been bound or chemically altered;

wherein said DNA is a nucleic acid that encodes a protein that inhibits or blocks said agent from binding to or chemically altering said preselected ligand

- 20 370. The method of claim 369, wherein said minicell is a eubacterial minicell, a poroplast, a spheroplast or a protoplast.
371. The method of claim 369, wherein said DNA has a nucleotide sequence that encodes the amino acid sequence of said protein that inhibits or blocks said agent from binding to or chemically altering said preselected ligand.
- 25 372. The method of claim 369, wherein a protein that binds or chemically alters said preselected ligand is a target protein for compounds that are therapeutic for a disease that is treated by administering said drug to an organism in need thereof.
373. A method of identifying an agent that effects the activity of a protein, comprising contacting a library of two or more candidate agents with a minicell comprising said protein or a polypeptide derived from said protein, assaying the effect of candidate
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WO 03/072014

PCT/US02/16877

agents on the activity of said protein, and identifying agents that effect the activity of said protein.

374. The method of claim 373, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.

5 375. The method of claim 373, wherein said protein or said polypeptide derived from said protein is displayed on the surface of said minicell.

376. The method of claim 373, wherein said protein is a membrane protein.

377. The method of claim 376, wherein said membrane protein is selected from the group consisting of a receptor, a channel protein and an enzyme.

10 378. The method of claim 373, wherein said activity of a protein is a binding activity or an enzymatic activity.

379. The method of claim 373, wherein said library of compounds is a protein library.

380. The method of claim 379, wherein said protein library is selected from the group consisting of a phage display library, a phagemid display library, and a ribosomal display library.

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381. The method of claim 373, wherein said library of compounds is a library of aptamers.

382. The method of claim 373, wherein said library of compounds is a library of small molecules.

20 383. A method of identifying an agent that effects the activity of a protein domain containing a library of two or more candidate agents with a minicell displaying a membrane fusion protein, said fusion protein comprising a first polypeptide, said first polypeptide comprising at least one transmembrane domain or at least one membrane anchoring domain, and a second polypeptide, wherein said second polypeptide comprises said protein domain.

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384. A method of identifying undesirable side-effects of a biologically active compound that occur as a result of binding of said compound to a protein, wherein binding a compound to said protein is known to result in undesirable side effects, comprising contacting a minicell that comprises said protein to said biologically active compound.

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WO 03/072014

PCT/US02/16877

385. The method of claim 384, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.
386. The method of claim 384, further comprising characterizing the binding of said biologically active compound to said protein.
- 5 387. The method of claim 384, further comprising characterizing the effect of said biologically active compound on the activity of said protein.
388. A method for identifying an agent that effects the interaction of a first signaling protein with a second signaling protein, comprising
- 10 (a) contacting a library of compounds with a minicell, wherein said minicell comprises:
- (i) a first protein comprising said first signaling protein and a first trans-acting regulatory domain;
- (ii) a second protein comprising said second signaling protein and a second trans-acting regulatory domain; and
- 15 (iii) a reporter gene, the expression of which is modulated by the interaction between said first trans-acting regulatory domain and said second trans-acting regulatory domain; and
- (b) detecting the gene product of said reporter gene.
389. The method of claim 388, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.
- 20 390. The method of claim 388, wherein said trans-acting regulatory domains are transactivation domains.
391. The method of claim 388, wherein said trans-acting regulatory domains are transrepression domains.
- 25 392. The method of claim 388, wherein said reporter gene is induced by the interaction of said first trans-acting regulatory domain and said second trans-acting regulatory domain.
393. The method of claim 388, wherein said agent that effects the interaction of said first signaling protein with said second signaling protein is an agent that causes or
- 30 promotes said interaction.

WO 03/072014

PCT/US02/16877

394. The method of claim 388, wherein said reporter gene is repressed by the interaction of said first trans-acting regulatory domain and said second trans-acting regulatory domain.
395. The method of claim 394, wherein said agent that effects the interaction of said first signaling protein with said second signaling protein is an agent that inhibits or blocks said interaction.
396. The method of claim 388, wherein said first signaling protein is a GPCR.
397. The method of claim 396, wherein said GPCR is an Edg receptor or a ScAMPER.
398. The method of claim 396, wherein said second signalling protein is a G-protein..
399. The method of claim 398, wherein said G-protein is selected from the group consisting of G-alpha-i, G-alpha-s, G-alpha-q, G-alpha-12/13 and Go.
400. The method of claim 388, wherein said library of compounds is a protein library.
401. The method of claim 400, wherein said protein library is selected from the group consisting of a phage display library, a phagemid display library, and a ribosomal display library.
402. The method of claim 388, wherein said library of compounds is a library of aptamers.
403. The method of claim 388, wherein said library of compounds is a library of small molecules.
404. A method for identifying an agent that effects the interaction of a first signaling protein with a second signaling protein, comprising contacting a library of two or more candidate agents with a minicell, wherein said minicell comprises:
- (a) a first fusion protein comprising said first signaling protein and a first detectable domain; and
  - (b) a second fusion protein comprising said second signaling protein and a second detectable domain,
- wherein a signal is generated when said first and second signaling proteins are in close proximity to each other, and detecting said signal.
405. The method of claim 404, wherein said signal is fluorescence.

WO 03/072014

PCT/US02/16877

406. The method of claim 404, wherein said first detectable domain and said second detectable domain are fluorescent and said signal is generated by FRET.
407. The method of claim 406, wherein said first and second detectable domains are independently selected from the group consisting of a green fluorescent protein, a blue-shifted green fluorescent protein, a cyan-shifted green fluorescent protein; a red-shifted green fluorescent protein; a yellow-shifted green fluorescent protein, and a red fluorescent protein, wherein said first fluorescent domain and said second fluorescent domain are not identical.
408. A method of bioremediation, said method comprising contacting a composition that comprises an undesirable substance with a minicell, wherein said minicell alters the chemical structure and/or binds said undesirable substance.
409. A method of bioremediation, said method comprising contacting a composition that comprises an undesirable substance with a minicell, wherein said minicell comprises an agent that alters the chemical structure of said undesirable substance.
410. The method of claim 409, wherein said agent that alters the chemical structure of said undesirable substance is an inorganic catalyst.
411. The method of claim 409, wherein said agent that alters the chemical structure of said undesirable substance is an enzyme.
412. The method of claim 411, wherein said enzyme is a soluble protein contained within said minicell.
413. The method of claim 412, wherein said soluble protein is selected from the group consisting of an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, a ligase and a synthetase.
414. The method of claim 411, wherein said enzyme is a secreted protein.
415. The method of claim 414, wherein said secreted protein is selected from the group consisting of an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, a ligase and a synthetase.
416. The method of claim 411, wherein said enzyme is a membrane protein.
417. The method of claim 416, wherein said membrane enzyme is selected from the group consisting of a cytochrome P450, an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, a ligase and a synthetase.

WO 03/072014

PCT/US02/16877

418. The method of claim 409, wherein said agent that alters the chemical structure of said undesirable substance is a fusion protein comprising a first polypeptide that comprises a transmembrane domain or at least one membrane-anchoring domain, and a second polypeptide, wherein said second polypeptide is an enzyme moiety.
- 5 419. The method of claim 418, wherein said second polypeptide is a polypeptide derived from a protein selected from the group consisting of an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, a ligase and a synthetase.
420. A method of bioremediation, said method comprising contacting a composition that comprises an undesirable substance with a minicell, wherein said minicell comprises an agent that binds an undesirable substance.
- 10 421. The method of claim 420, wherein said undesirable substance binds to and is internalized by said minicell or is otherwise inactivated by selective absorption.
422. The method of claim 420, wherein said agent that binds said undesirable substance is a secreted soluble protein.
- 15 423. The method of claim 422, wherein said secreted protein is a transport accessory protein.
424. The method of claim 420, wherein said agent that binds said undesirable substance is a membrane protein.
425. The method of claim 420, wherein said undesirable substance is selected from the group consisting of a toxin, a pollutant and a pathogen.
- 20 426. The method of claim 420, wherein said agent that binds said undesirable substance is a fusion protein comprising a first polypeptide that comprises a transmembrane domain or at least one membrane-anchoring domain, and a second polypeptide, wherein said second polypeptide is a binding moiety.
- 25 427. The method of claim 426, wherein said binding moiety is selected from the group consisting of an antibody, an antibody derivative, the active site of a non-enzymatically active mutant enzyme, a single-chain antibody and an aptamer.
428. A minicell-producing parent cell, wherein said parent cell comprises one or more of the following:
- 30 (a) an expression element that comprises a gene operably linked to expression sequences that are inducible and/or repressible, wherein induction or

WO 03/072014

PCT/US02/16877

repression of said gene regulates the copy number of an episomal expression construct;

- (b) a mutation in an endogenous gene, wherein said mutation regulates the copy number of an episomal expression construct;
  - 5 (c) an expression element that comprises a gene operably linked to expression sequences that are inducible and/or repressible, wherein induction or repression of said gene causes or enhances the production of minicells; and
  - (d) a mutation in an endogenous gene, wherein said mutation causes or enhances minicell production.
- 10 429. The minicell-producing parent cell of claim 428, further comprising an episomal expression construct.
430. The minicell-producing parent cell of claim 428, further comprising a chromosomal expression construct.
431. The minicell-producing parent cell of claim 429, wherein said expression sequences  
15 of said expression construct are inducible and/or repressible.
432. The minicell-producing parent cell of claim 428, wherein said minicell-producing parent cell comprises a biologically active compound.
433. The minicell of claim 428 wherein said gene that causes or enhances the production of minicells has a gene product that is involved in or regulates DNA replication,  
20 cellular division, cellular partitioning, septation, transcription, translation, or protein folding.
434. A minicell-producing parent cell, wherein said parent cell comprises an expression construct, wherein said expression construct comprises expression sequences operably linked to an ORF that encodes a protein, and a regulatory expression element,  
25 wherein said regulatory expression element comprises expression sequences operably linked to a regulatory gene that encodes a factor that regulates the expression of said ORF.
435. The minicell-producing parent cell of claim 434, wherein said expression sequences of said expression construct are inducible and/or repressible.
- 30 436. The minicell-producing parent cell of claim 434, wherein said expression sequences of said regulatory expression construct are inducible and/or repressible.

- 397

WO 03/072014

PCT/US02/16877

450. The minicell of claim 458, wherein said binding moiety is a single-chain antibody.
451. The minicell of claim 458, wherein said binding moiety is directed to a ligand selected from the group consisting of an epitope displayed on a pathogen, an epitope displayed on an infected cell and an epitope displayed on a hyperproliferative cell.
- 5 452. The minicell of claim 458, wherein said biologically active compound is selected from the group consisting of a radioisotope, a polypeptide, a nucleic acid and a small molecule.
453. The minicell of claim 448, wherein a ligand binds to and is internalized by said minicell or is otherwise inactivated by selective absorption.
- 10 454. A pharmaceutical composition comprising the minicell of claim 448.
455. A method of reducing the free concentration of a substance in a composition, wherein said substance displays a ligand specifically recognized by a binding moiety, comprising contacting said composition with a minicell that displays said binding moiety, wherein said binding moiety binds said substance, thereby reducing the free concentration of said substance in said composition.
- 15 456. The method of claim 455, wherein said minicell is selected from the group consisting of a eubacterial minicell, a poroplast, a spheroplast and a protoplast.
457. The method of claim 455, wherein said substance is selected from the group consisting of a nucleic acid, a lipid, a polypeptide, a radioactive compound, an ion and a small molecule.
- 20 458. The method of claim 455, wherein said binding moiety is selected from the group consisting of an antibody, an antibody derivative, a channel protein and a receptor.
459. The method of claim 455, wherein said composition is present in an environment.
460. The method of claim 459, wherein said environment is water, air or soil.
- 25 461. The method of claim 455, wherein said composition is a biological sample from an organism.
462. The method of claim 461, wherein said biological sample is selected from the group consisting of blood, serum, plasma, urine, saliva, a biopsy sample, feces, tissue and a skin patch.

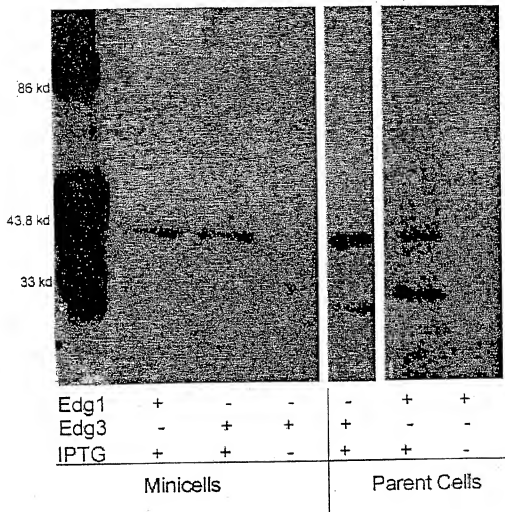
**WO 03/072014****PCT/US02/16877**

463. The method of claim 461, wherein said substance binds to and is internalized by said minicell or is otherwise inactivated by selective absorption.
464. The method of claim 463, wherein said biological sample is returned to said organism after being contacting to said minicell.

WO 03/072014

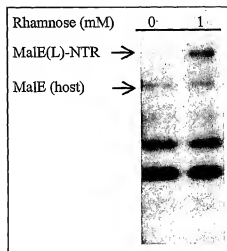
PCT/US02/16877

1/2



WO 03/072014

PCT/US02/16877

**2/2****Figure 2**

WO 03/072014

PCT/US02/16877

# SEQUENCE LISTING

## SEQ ID NO 1

5 pMPX-23 (complete *ftsZ* cloned into pMPX-18 using PCR-introduced PstI and XbaI)

		Shine-Delgarno	PstI
10	1621 1	CCATACCCGTTTTTTTGGGCTAGCAGGAGGAATCACCTGCAGATGTTTGAACCAATGG	M F E P M
	1681 6	AACCTACCAATGACGCGGTGATTAAAGTCATCGGCGTCGGCGCGGCGGCGTAATGCTG	E L T N D A V I K V I G V G G G G G N A
15	1741 26	TTGAACACATGGTGCGCGAGCGCATTTGAAGTGTTGAATTCCTCGCGGTAATACCGATG	V E H M V R E R I E G V E F F A V N T D
	1801 46	CACAAGCGCTGCGTAAAAACAGCGGTTGGACAGACGATTCAAATCGGTAGCGGTATCACCA	A Q A L R K T A V G Q T I Q I G S G I T
20	1861 66	AAGGACTGGGCGCTGGCGCTAATCCAGAAGTTGGCGCAATGCGGCTGATGAGGATCGCG	K G L G A G A N P E V G R N A A D E D R
25	1921 86	ATGCATTCGCGTCGCGCGCTGGAAGGTGCAGACATGGTCTTTATTGCTCGCGGTATGGGTG	D A L R A A L E G A D M V F I A A G M G
	1981 106	GTGGTACCGGTACAGGTGCAGCACCACTGCTCGCTGAAGTGGCAAAGATTGGGTATCC	G G T G T G A A P V V A E V A K D L G I
30	2041 126	TGACCGTGTGCTGCTCACTAAGCCTTTCAACTTTGAAGGCAAGAAGCGTATGGCATTCG	L T V A V V T K P F N F E G K K R M A F
	2101 146	CGGAGCAGGGGATCACTGAACCTGTCCAAAGCATGTGGACTCTCTGATCACTATCCGAAACG	A E Q G I T E L S K H V D S L I T P N
35	2161 166	ACAAATGCTGAAAGTTCTGGGCGCGGTATCTCCTGCTGGAATGCGTTTGGCGCAGCGA	D K L L K V L G R G I S L L D A F G A A
40	2221 186	ACGATGTACTGAAAGCGCTGTGCAAGGTATCGCTGAACTGATTACTCGTCCGGGTTTGA	N D V L K G A V Q G I A E L I T R P G L
	2281 206	TGAACGTGGACTTTGACAGCGTACGACCGTAATGTCTGAGATGGGCTACGCAATGATGG	M N V D F A D V R T V M S E M G Y A M M
45	2341 226	GTTCTGGCGTGGCGAGCGGTGAAGACCGTGCAGGAAGCTGCTGAANTGGCTATCTCTT	G S G V A S G E D R A E E A A E M A I S
	2401 246	CTCGCTGCTGGAAGATATCGACCTGTCTGGCGCGCGCGGCTGCTGGTTAAACATCACGG	S P L L E D I D L S G A R G V L V N I T
50	2461 266	CGGGCTTCGACCTCGCTCTGGATGAGTTGGAACCGTAGGTAAACCATCCGTGCAATTTG	A G F D L R L D E F E T V G N T I R A F
55	2521 286	CTTCGACAACGCGACTGTGGTTATCGGTACTTCTCTTGACCCGGATATGAATGACGAGC	A S D N A T V V I G T S L D P D M N D E
	2581	TGCGCTAACCGTTGTGCGACAGGTATCGCATGGACAAACGTCTCTGAAATCACTCTGG	

WO 03/072014

PCT/US02/16877

306 L R V T V V A T G I G M D K R P E I T L  
2641 TGACCAATAAGCAGGTTTCAGCAGCCAGTGGATCGCTACCGCAGCATGGGATGGCTC  
326 V T N K Q Q V Q Q P V M D R Y Q Q H G M A  
5 2701 CGCTGACCCAGGAGCAGAAGCCGGTTGCTAAAGTCGTGAATGACAATGCGCGCAAACTG  
346 P L T Q E Q K P V A K V N N D N A P Q T  
2761 CGAAAGAGCCGGATTATCTGGATATCCAGCATTCCTGCGTAAGCAAGCTGATATAATAA  
10 366 A K E P D Y L D I P A F L R K Q A D  
XbaI  
2821 CTAGAGGATCCCCGGTACCGAGCTCGAATTCGTAATCATGGTCATAGCTGTTCTCTGTG

15 Sequence contains full-length *ftsZ* PCR amplified from *E. coli* MG1655 using oligos containing PstI and XbaI restriction sites.

20 SEQ ID NO 2

pMPX-47 (complete *ftsZ* cloned into pMPX-5 using PCR-introduced PstI and XbaI)

25 2401 GAATTTCAGGCGCTTTTTCAGTGGTCTGAATGAAATTCAGGAGTACATCTTCGAGAT  
Shine-Delgarno PstI  
M  
2461 GTTTGAACCAATGGAACCTTACCAATGACGCGGTGATTAAAGTCATCGGCGTGGCGGCGG  
30 2 F E P M E L T N D A V I K V I G V G G G  
2521 CGGCGGTAAATGCTGTTGAACACATGCTGCGCGAGCGCATTGAAGGTGTTGAATCTTCGC  
22 G G N A V E H M V R E R I E G V E F F A  
35 2581 GGTAAATACCGATGCACAAGCGCTGCGTAAACAGCGGTTGGACAGAGATTCAAATCGG  
42 V N T D A Q A L R K T A V G Q T I Q I G  
2641 TAGCGGTATCACCAAAGGACTGGGCGCTGGCGCTAATCCAGAAGTTGGCGCAATGCGGC  
62 S G I T K G L G A G A N P E V G R N A A  
40 2701 TGATGAGGATCGCGATGCATTGCGTGGCGCTGGAAGGTGCAGACATGTTCTTTATTGC  
82 D E D R D A L R A A L E G A D M V F I A  
2761 TCGGGGTATGGTGGTGGTACCGGTACAGGTGCAGCACCAGTCTGCTGCTGAAGTGGCAA  
45 A G M G G G T G T G A A P V V A E V A K  
2821 AGATTGGGTATCTTGACCGTTGCTGCTCACTAAGCCTTTCAACTTTGAAGCAAGAA  
122 D L G I L T V A V V T K P F N F E G K K  
50 2881 GCGTATGGCATTTCGCGAGCAGGGGATCACTGAAGTTCGAAGCATGTGGACTCTCTGAT  
142 R M A F A E Q G I T E L S K H V D S L I  
2941 CACTATCCGAACGACAAACTGCTGAAGTTCTGGGCGCGGTTATCTCCCTGCTGGATGC  
162 T I P N D K L L K V L G R G I S L L D A  
55 3001 GTTTGGCGCAGCGAAGCATGTACTGAAGGCGCTGTGCAAGGTATCGCTGAATGATTC  
182 F G A A N D V L K G A V Q G I A E L I T  
3061 TCGTCCGGGTTTGATGAACGTGGACTTTGCAGACGTACGCACCCTGAATGTCTGAGATGGG

PCT/US02/16877

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PCT/US02/16877

4/268

PCT/US02/16877

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	<i>intD</i> homology for recombination			Stop <i>rhaR</i>	
181	AAGCTGTCAT	TGCGGCGCTT	CAGTCTCCGC	TGCATACTGT	CCTTAATCTT
	TCCTGCAATAT				
241	GAGTAGACGC	CACTGGCTGG	GCCTCATCCG	GGTTTCCCGG	GTAACAACCA
50	CCGAAAAATA				
301	GTTACTATCT	TCAAAGCCAC	ATTGGTCTGA	AATATCACTG	ATTACAGGC
	GGCTATGCTG				
361	GAGAAGATAT	TGCGCATGAC	ACACTCTGAC	CTGTGCGAGA	TATTTGATTGA
	TGGTCAATCC				
421	AGTCTGCTGG	CGAAATTGCT	GACGCAAAAC	GCCTCACTG	CACGATCCCT
55	CATCACAAAA				
481	TTTATCCAGC	GCAAAGGGAC	TTTTCAGGCT	AGCGCCACG	CGGGTAATCA
	GCTTATCCAG				

WO 03/072014

PCT/US02/16877

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541   CAACGTTTCG CTGGATGTTG GCGGCAACGA ATCACTGGTG TAACGATGSC
GATTACAGCAA
601   CATCACCAAC TGCCCCAACA GCAACTCAGC CATTTTCGTTA GCAACCGCA
CATGCTGACT
5   661   ACTTTCATGC TCAAGCTGAC CGATAACCTG CCGCGCCTGC GCCATCCCCA
TGCTACCTAA
721   GCGCCAGTGT GGTGCCCCTG CGCTGGCGTT AAATCCCGGA ATCGCCCCCT
GCCAGTCAAG
10  781   ATTCAGCTTC AGACGCTCCG GGCAATAAAT AATATTCTGC AAAACCAGAT
CGTTAACGGA
841   AGCGTAGGAG TGTTTATCGT CAGCATGAAT GTAAAAGAGA TCGCCACGGG
TAATGCGATA
15  901   ACGGCGATCG TTGAGTACAT GCAGGCCATT ACCGCGCCAG ACAATCACCA
GCTCACAAAA
961   ATCATGTGTA TGTTTACGAA AGACATCTTG CGGATAACGG TCAGCCACAG
CGACTGCCCTG
1021  CTGGTCGCTG GCAAAAAAAT CATCTTTTGA AAGTTTAAAC TGATGCGCCA
CCGTGGCTAC
1081  CTGGGCCAGA GAACGAAGTT GATTATTGCG AATATGGCGT ACAAATACGT
20  TGAGAAGATT

      Stop rhaS      Start rhaR
1141  CGCGTTATTG CAGAAAGCCA TCCCGTCCCT GGCGAATATC ACGCGTGAC
25  CAGTTAAACT      ←

1201  CTCGGCGAAA AAGCGTCGAA AAGTGGTTAC TGTGCTGAA TCCACAGCGA
TAGGCGATGT
1261  CAGTAACGCT GGCGTCGCTG TGGCGTAGCA GATGTCGGGC TTTCATCAAT
30  CGCAGGCGGT
1321  TCAGGTATCG CTGAGGCGTC AGTCCCGTTT GCTGCTTAAG CTGCCGATGT
AGCGTACGCA
1381  GTGAAAGAGA AAATTGATCC GCCACGGCAT CCAATTTCAC CTCATCGGCA
AAATGGTCTT
35  1441  CCAGCCAGGC CAGAAGCAAG TTGAGACGTG ATGCGCTGTT TTCCAGGTTT
TCCTGCCAAC
1501  TGCTTTTACG CAGCAAGAGC AGTAATTGCA TAAACAAGAT CTCGCGACTG
GCGGTCGAGG
40  1561  GTAAATCATT TTCCCTTCC TGCTGTTCOA TCTGTGCAAC CAGCTGTCCG
ACCTGCTGCA
1621  ATACGCTGTG GTTAAACGCGC CAGTGAGACG GATACTGCCC ATCCAGCTCT
TGTGGCAGCA
1681  ACTGATTTCAG CCCGGCGAGA AACTGAAATC GATCCGGCGA GCGATACAGC
ACATTGGTCA
45  1741  GACACAGATT ATCGGTATGT TCATACAGAT GCCGATCATG ATCGCGTATG
AAACGAGCCG
1801  TGCCACCCTG GATGGTATAG GCGTGCCCAT TAAACACATG AATACCCCTG
CCATGTTGCA
1861  CAATCACAAT TTCATGAAAA TCATGATGAT GTTCAGGAAA ATCGCGCTGC
50  GGGAGCCGGG

      Start rhaS
1921  GTTCTATCGC CACGGACGCG TTACCAGACG GAAAAAATC CACACTATGT
55  AATACGGTCA

      ←

1981  TACTGGCCTC CTGATGTCGT CAACACGGCG AAATAGTAAT CACGAGTCA
60  GGTCTTACC

```

WO 03/072014

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2041 TTAAATTTTC GACGGAAAAC CACGTAAAAA ACGTCGATTT TTCAAGATAC  
AGCGTGAATT  
5 2101 TTCAGGAAAT GCGGTGAGCA TCACATCACC ACAATTCAGC AAATTGTGAA  
CATCATCAGC  
2161 TTCATCTTTT CCTGGTTGCC AATGGCCCAT TTTCCTGTCA GTAACGAGAA  
GGTCGCAAT

Shine-Delgarno Start *ftsZ*  
10 2221 TCAGGCGCTT TTAGACTGG TCGTAATGAA ATTCAGCAG ATCACATATG  
TTTGACCAA  
→

2581 TGGAACTTAC CAATGACGCG GTGATTAAAG TCATCGCGCT CGCGCGCGCG  
15 GCGGGTAATG  
2641 CTGTTGAACA CATGGTGCAG GAGCGCATTG AAGGTGTTGA ATTCTTCGCG  
GTAAATACCG  
2701 ATGCACAAGC GCTGCGTAAA ACAGCGGTTG GACAGACGAT TCAAAATCGGT  
AGCGGTATCA  
20 2761 CCAAAGGACT GGGCGCTGGC GCTAATCCAG AAGTTGGCCG CAATGCGGCT  
GATGAGGATC  
2821 GCGATGCAAT GCGTGCAGCG CTGGAAGGTG CAGACATGGT CTTTATGTCT  
GCGGGTATGG  
2881 GTGGTGGTAC CGGTACAGGT GCAGCACCAG TCGTCGCTGA AGTGGCAAAA  
25 GATTGGGTA  
2941 TCCTGACCGT TGCTGTCTGC ACTAAGCCCTT TCAACTTTGA AGGCAAGAG  
CGTATGGCAT  
3001 TCGCGGAGCA GGGGATCACT GAACTGTCCA AGCATGTGGA CTCTCTGATC  
ACTATCCCGA  
30 3061 ACGACAAAT GCTGAAAGTT CTGGGCGCGG GTATCTCCCT GCTGGATGCG  
TTTGGCGCAG  
3121 CGAACGATGT ACTGAAAGGC GCTGTGCAAG GTATCGCTGA ACTGATTACT  
CGTCGGGTT  
3181 TGATGAACGT GGACTTTGCA GACGTACGCA CCGTAATGTC TGAGATGGGC  
35 TACGCAATGA  
3241 TGGGTTCTGG CGTGGCGAGC GGTGAAGACC GTGCGGAAGA AGCTGCTGAA  
ATGGCTATCT  
3301 CTGCTCCGCT GCTGGAAGAT ATCGACCTGT CTGGCGCGCG CGGCGTGTCT  
GTTAACATCA  
40 3361 CGGCGGGCTT CGACCTGCGT CTGGATGAGT TCGAAACGGT AGGTAAACACC  
ATCCGTGCAT  
3421 TTGCTTCCGA CAACGCGACT GTGGTTATCG GTACTTCTCT TGACCCGGAT  
ATGAATGACG  
3481 AGCTGCGCGT AACCGTTGTT GCGCAGGTA TCGGCATGGA CAAACGTCCT  
45 GAAATCACTC  
3541 TGGTGACCAA TAAGCAGGTT CAGCAGCCAG TGATGGATCG CTACCAGCAG  
CATGGGATGG  
3601 CTCCGCTGAC CAGGAGCAG AAGCCGGTTG CTAAAGTCGT GAATGACAAT  
50 GCGCCGCAAA

Stop

*ftsZ*  
3661 CTGCGAAAGA GCCGGATTAT CTGGATATCC CAGCATTCCT GCGTAAGCAA  
55 GCTGATTAAT

FRT scar  
3721 AATCTAGAGG CGTTACCAAT TATGACAACT TGACGGGAAG TTCCTATACT  
TTCTAGAGAA

60

intD homology for recombination

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3781 TAGGAACCTTC CCAAGCCAG TATCAACTCA GACAAAGGCA AAGCATCTTG

Bold, italicized represents homology between the PCR product shown below and *intD*.

5

*rhaRS::Prha::ftsZ::FRT::kan::Frt*

Following RED recombination into *intD*, the kanamycin cassette was removed with *flp* recombinase resulting in a single FRT scar as depicted above. Bold alone represents FRT scar after the *flp* reaction.

10

SEQ ID NO 5

15 *lacI::Ptac::ftsZ* inserted by RED recombination into *E. coli* MG1655  
*intD*

20 181 *intD* homology for recombination Stop *lacI*  
AAGCCTGCAT TCGGCGCTT CAGTCTCCGC TGCATACTGT CCTTAAATAA  
GTGAGTCGAT

241 ATTGTCTTTG TTGACCAGTA ATACCTTATG GAAACGGATA ATTCGCTTAT  
CCATATCTAC

25 301 GTCGGCCTTA CCCAGATTCT GCATTCTTAA TCCAGGCTTG ATCTCTTCAC  
CCTTCAGCAA

361 CGTGTCTGGC ACGGCTGCGA GTGCGTAACC TGCAGAGGCC GGATCGTAGAG  
TAATCCCTTC

421 GGTGATATCA CCACCTTTAA TCAGTGATGC CGCCTGTGAA GGGATCATCA  
TGCCATAGAC

30 481 TCGCAGCTTA TTTTTCGCCC GTTCTCTTTT CACCGCACGT CCGCGCCCAA  
TCGGACCGIT

541 TGAACCAAAG GAGACAACCG CTTTCAAGTC AGGATAGGTT TTCATCAGGT  
CCAGTGTAGT

35 601 ACGACGTGAG ACATCCACAC TCTCGGCAAC CGGCATGCGG CGGGTAACCTT  
CATGCATATC

661 CGGGTAATGC TCTTCTGGT ATTTCAACCAG CAGTCAGGCC CATAAGTTAT  
GCTGCGGCAC

721 GGTCAAACTA CCCACGTAAA TCACATAGCC GCCCTTGCCA CCCATGCGTT  
TCGCCATATG

40 781 CTCACATAT TCAGCGGCAA ATTTTTCGTT ATCAATGATT TCGATATCCC  
AGTTAGCACT

841 TGGGTGACCG GGGGATTCTG TGGTCAGAAC CACAATTCGG GCATCTCGCG  
CTTTTGTGAA

45 901 TACCGTTTCC AGCACGTTGG CATCGTTTGG CACGATAGTA ATTGCATTAA  
CCTTACGGGC

961 GATTAAATCC TCAATAATTT TAACTTGTG CGGAGCATCA GTACTTGAAG  
GCCCCACCTG

50 1021 TGAGGCATTA ACACCAAAGG CTTTACCCGC CTCACCCACA CCTTCGCCCA  
TGCGATTAAA

1081 CCACGGCATA CCATCGACTT TAGAAATATT CACCACGACT TTTTCCGCTG  
CCTGGAGCGG

1141 CGCAGAAATT AGCGCAGCGC CTAATAACAG CGAAGACACC ATATTGATAA  
CAAAACGTTT

55

Start *lacI* Start *ftsZ*  
1201 ATTCATCAT Ptac sequence (see reference below) A  
TGGAACCTTAC

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12 CAATGACGCG GTGATTAAAG TCATCGGCGT CGGCGGCGGC GGCGGTAATG  
 5 CTGTGAAACA  
 72 CATGGTGGCG GAGCGCATTG AAGGTGTTGA ATTCTTCGCG GTAAATACCG  
 ATGCACAAGC  
 132 GCTGCGTAAA ACAGCGGTTG GACGACGAT TCAAAATCGGT AGCGGTATCA  
 CCAAAGGACT  
 10 192 GGGCGCTGGC GCTAATCCAG AAGTTGGCCG CAATGCGGCT GATGAGGATC  
 GCGATGCGATT  
 252 GCGTGGCGCG CTGGAAGGTG CAGACATGGT CTTTATTGCT GCGGGTATGG  
 GTGGTGGTAC  
 312 CGGTACAGGT GCAGCACCAG TCGTCGCTGA AGTGGCAAAA GATTTGGGTA  
 15 TCCTGACCGT  
 372 TGCTGTGCTC ACTAAGCCTT TCAACTTTGA AGGCAAGAAG CGTATGGCAT  
 TCGCGGAGCA  
 432 GGGGATCACT GAACGTGCCA AGCATGTGGA CTCTCTGATC ACTATCCCGA  
 ACGACAAACT  
 20 492 GCTGAAAGTT CTGGGCCGCG GTATCTCCCT GCTGGATGCG TTTGGCGCAG  
 CGAACGATGT  
 552 ACTGAAAGGC GCTGTGCAAG GTATCGCTGA ACTGATTACT CGTCCGGGTT  
 TGATGAACGT  
 25 612 GGACTTTGCA GACGTACGCA CCGTAATGTC TGAGATGGGC TACGCAATGA  
 TGGGTTCTGG  
 672 CGTGGCGAGC GGTCAAGACC GTGCGGAAGA AGCTGCTGAA ATGGCTATCT  
 CTTCTCGCT  
 732 GCTGGAAGAT ATGCACTGTG CTGGCGCGCG CGGCGTGCTG GTTAACATCA  
 CGGCGGGCTT  
 30 792 CGACCTCGCT CTGGATGAGT TCGAAACGGT AGGTAACACC ATCCGTGCAT  
 TTGCTTCCGA  
 852 CAACGCGACT GTGGTTATCG GTACTTCTCT TGACCCGGAT ATGAATGACG  
 AGCTGCGCGT  
 912 AACCGTTGTT GCGACAGGTA TCGGCATGGA CAAACGTCTT GAAATCACTC  
 35 TGGTGACCAA  
 972 TAAGCAGGTT CAGCAGCCAG TGATGATCG CTACCAGCAG CATGGGATGG  
 CTCCGCTGAC  
 1032 CCAGGAGCAG AAGCCGGTTG CTAAGTCTGT GAATGACAAAT GCGCCGCAAA  
 CTGCGAAAGA  
 40 1092 GCCGATTAT CTGGATATCC CAGCATTCCT GCGTAAGCAA GCTGATTAAAT  
 AATCTAGAGG  
 1152 CGTTACCAAT TATGACAACT TGACGGGAAG TTCTATTCT CTAGAAAGTA  
 TAGGAACTTC  
 1212 CCAAAGCCAG TATCAACTCA GACAAAGGCA AAGCATCTTG

45

Bold, italicized represents homology between the PCR product shown below and *intD*.

*lacI::Ptac::ftsZ::FRT::kan::Frt*

50

Following RED recombination into *intD*, the kanamycin cassette was removed with *flp* recombinase resulting in a single FRT scar as depicted above.

Garrido, T., et al. 1993. Transcription of *ftsZ* oscillates during the cell cycle of *Escherichia coli*. EMBO J. 12:3957-3965

55

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pMPX-5 expression vector

←

WO 03/072014

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1381 AACTCTCGGC GAAAAAGCGT CGAAAAGTGG TTAAGTCTGC TGAATCCACA  
GCATAGGCG  
1441 ATGTTCAGTAA CGCTGGCCTC GCTGTGGCGT AGCAGATGTC GGGCTTTTCA  
CAGTCGAGG  
5 1501 CGGTTCAGGT ATCGCTGAGG CGTCAGTCCC GTTGTCTGCT TAAGCTGCGG  
ATGTAGCTA  
1561 CGCAGTGAAA GAGAAAATTG ATCCGCCACG GCATCCCAAT TCACCTCATC  
GGCAAAATGG  
1621 TCCTCCAGCC AGGCCAGAAG CAAGTTGAGA CGTGATGCGC TGTTTTCCAG  
10 GTTCTCCTGC  
1681 AACTGCTTTT TAOCGAGCAA GAGCAGTAAT TGCATAAACA AGATCTCGCG  
ACTGGCGGTC  
1741 GAGGGTAAAT CATTTTCCCC TTCTGCTGT TCCATCTGTC CAACCAAGTC  
TCGCACTGTC  
1801 TGCAATACGC TGTGGTTAAC GCGCCAGTGA GACGGATACT GCCCATCCAG  
15 CTCTTGTGGC  
1861 AGCAACTGAT TCAGCCCGGC GAGAACTGA AATCGATCCG GCGAGCGATA  
CAGCATTG  
1921 GTCAGACACA GATTATCGGT ATGTTTCATC AGATGCCGAT CATGATCGCG  
20 TAGCAACACG  
1981 ACCGTGCCAC CGGTGATGGT ATAGGGCTGC CCATTAAACA CATGAATACC  
CGTGCCATGT  
2041 TCGACAACTCA CAATTTCATG AAAATCATGA TGATGTTGAG GAAATCCGCG  
CTGCGGGAGC  
25 2101 CGGGGTCTTA TCGCCACGGA CGGTTTACCA GACGGAAAAA AATCCCACT  
ATGTAATACG

Start rhas

2161 GTCATACTGG CCTCTGATG TCGTCAACAC GCGGAAATAG TAATCACGAG  
30 GTCAGGTTCT

←

2221 TACCTTAAAT TTTCGACGGA AAACCACGTA AAAAACGTCG ATTTTTCAAG  
ATACAGCGTG  
35 2281 AATTTTCAGG AAATGCGGTG AGCATCATAT CACCACAATT CAGCAAAATTG  
TGAACATCAT  
2341 CACGTTTCATC TTTCCTGGT TGCCAATGGC CCATTTTCTCT GTCAGTAACG  
AGAAGGTGCG

40 a.

Shine-Delgarno

PstI

2401 GAATTCAGGC GCTTTTTAGA CTGGTCGTAA TGAAATTCAG CAGGATCACA  
TTCTGCAGGT

→

45 SalI XbaI BamHI KpnI  
2461 CGACTCTAGA GGATCCCGCG GTACCGAGCT CGAATTCGTA ATCATGGTCA  
TAGCTGTTTC  
2521 CTGTGTGAAA TTGTTATCCG CTCACAAATC CACACAACAT ACGAGCCGGA  
50 AGCATRAAGT  
2581 GTAAGCCCTG GGGTGCCTAA TGAGTGAGCT AACTCACAAT AATTCGCTTG  
CGTCACTGC  
2641 CCGCTTTCCA GTCCGGGAAC GTGTCGTGCC AGCTGCATTA ATGAATCCGC  
CRACGCGCG  
55 2701 GGAGAGGCGG TTTGCGTATT GGGCGCTCTT CCGCTTCTCT GCTCACTGAC  
TGGCTGCGCT  
2761 CGTCTGCTTCG GCTGCGGCGA GCGGTATCAG CTCACTCAAA GCGGTAAATA  
CGGTTATCCA

WO 03/072014

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2821 CAGAATCAGG GGATAACGCA G3AAAGAACA TGTGAGCAAA AGGCCAGCAA  
AAGGCCAGGA  
2881 ACCGTAAAAA GGGCGCGTTG CTGGCGTTTT TCCATAGGCT CGCCCCCCT  
GACGAGCATC  
5 2941 ACAAAAATCG ACGCTCAAGT CAGAGGTGGC GAAACCCGAC AGGACTATAA  
AGATACCAGG  
3001 CGTTTCCCCC TGGAAGCTCC CTCGTGCGCT CTCCTGTTCG GACCCGTCCG  
CTTACCGGAT  
10 3061 ACGTGTCCGC CTTTCTCCCT TCGGGAAGCG TGGCGCTTTC TCATAGCTCA  
CGCTGTAGGT  
3121 ATCTCAGTTC GGTGTAGGTC GTTCGCTCCA AGCTGGGCTG TGTGCACGAA  
CCCCCGTTC  
3181 AGCCCGACCG CTGCGCCTTA TCCGTTAACT ATCGTCTTGA GTCCAACCG  
GTAAGACACG  
15 3241 ACTTATCGCC ACTGGCAGCA GCCACTGGTA ACAGGATTAG CAGAGCGAGG  
TATGTAGGCG  
3301 GTGCTACAGA GTTCTTGAAG TGGTGGCCTA ACTACGGCTA CACTAGAAGG  
ACAGTATTTG  
3361 GTATCTGCGC TCTGCTGAAG CCAGTTACCT TCGGAAAAAG AGTTGGTAGC  
20 TCTTGATCCG  
3421 GCAAAACAAAC CACCGCTGGT AGCGGTGGTT TTTTGTGTTG CAAGCAGCAG  
ATTACCGCAC  
3481 GAAAAAAAGG ATCTCAAGAA GATCCTTTGA TCTTTTCTAC GGGGTCTGAC  
GCTCAGTGA  
25 3541 AC3AAAATC ACGTTAAGGG ATTTTGGTCA TGAGATTATC AAAAAGATC  
TTCACCTAGA  
3601 TCCTTTTAAA TTAAAAATGA AGTTTTAAAT CAATCTAAAG TATATATGAG  
TAAACTTGGT  
30 Stop bla  
3661 CTGACAGTTA CCAATGCTTA ATCAGTGAGG CACCTATCTC AGCGATCTGT  
CTATTTGGTT  
3721 CATCCATAGT TGCTGACTC CCGTCTGTGT AGATAACTAC GATACGGGAG  
35 GGCTTACCAT  
3781 CTGGCCCCAG TGCTGCAATG ATACCGCGAG ACCCAGGCTC ACCGGCTCCA  
GATTTATCAG  
3841 CAATAAAACCA GCCAGCCGGA AGGGCCGAGC GCAGAAGTGG TCCTGCAACT  
TTATCCGCCT  
40 3901 CCATCCAGTC TATTAATTGT TGCGGGGAAG CTAGAGTAAG TAGTTGCGCA  
GTTAATAGTT  
3961 TGCGCAACGT TGTTGCCATT GCTACAGGCA TCGTGGTGTG ACGCTCGTGG  
TTTGGTATGG  
45 4021 CTTCAATCAG CTCGGTTCC CAACGATCAA GCGGAGTTAC ATGATCCCCC  
ATGTTGTGCA  
4081 AAAAAGCGGT TAGTCTCTTC GGTCTCTCCA TCGTTGTGAG AAGTAAGTTG  
GCCGCAGTGT  
4141 TATCACTCAT GGTATGGCA GCACTGCATA ATTCTCTTAC TGTATGCGCA  
TCGGTAAGAT  
50 4201 GCTTTTCTGT GACTGCTGAG TACTCAACCA AGTCATTCTG AGAATAGTGT  
ATGCGGCGAC  
4261 CGAGTTGCTC TTGCCCCGGC TCAATACGGG ATAATACCGC GGCACATAGC  
AGAACTTTAA  
4321 AAGTGCTCAT CATTTGAAAA CGTTCTTCGG GCGGAAAACT CTCAGGATC  
55 TTACCGCTGT  
4381 TGAGATCCAG TTCGATGTAA CCCACTCTGT CACCCAACTG ATCTTCAGCA  
TCTTTTACTT  
4441 TCACCAGCGT TTCTGGGTGA GCAAAAACAG GAAGGCAAAA TGCCGCCAAAA  
AAGGGAATAA  
60

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The segment *rhaR* through the *Prha* control region was taken from the *E. coli* MG1655 chromosome using PCR-added *Hind*III and *Pst*I restriction sites. This fragment was cut with *Hind*III and *Pst*I and cloned into pUC-18 cut with the same enzymes. Italicized sequence constitutes both *rhaSR* and protein to be expressed promoter region.

pMPX-32 (*ΔphoA* cloned into pMPX-5 using PCR-introduced PstI and XbaI)

13/268

PCT/US02/16877

$\Delta phoA$  sequence constitutes *phoA* residues 49-453.

SEO ID NO 8

[illegible]

WO 03/072014

PCT/US02/16877

2641 TACTGCACCCGGCGGTGCTCGCCGTTTAAACGGGTGATCAGACTGCCGCTCTGCGTGATTC  
62 T A P G G A R R L T G D Q T A A L R D S

5 2701 TCTTAGCGATAAACCTGCAAAAAATATTATTTTGTCTGATTTGGCGATGGGAGACTC  
82 L S D K P A K N I I L L I G G D G M G D S

2761 GGAATATTACTCCCGCACGTAATTATGCCGAAGGTGCGGCGCGCTTTTTTAAAGGTATAGA  
102 E I T A A R N Y A E G A G G F F K G I D

10 2821 TGCCTTACCGCTTACCGGGCAATACACTCACTATGCGCTGAATAAAAAACCGGCAAAAC  
122 A L P L T G Q Y T H Y A L N K K T G K P

2861 GGACTACGTACCGACTCGCTGCATCAGCAACCGCTGGTCAACCGGTGTCAAAACCTA  
142 D Y V T D S A A S A T A W S T G V K T Y

15 2941 TAACGGCGCGCTGGGCGTGCATATTACGAAAAAGATCACCAACGATTCTGGAATGGC  
162 N G A L G V D I H E K D H P T I L E M A

3001 AAAAGCCGACGCTCTGGCGACCGGTAAACGTTTCTACCGCAGAGTTGCAGGATGCCACGCC  
182 K A A G L A T G N V S T A E L Q D A T P

3061 CGCTGCGCTGGTGGCACATGTGACCTCGCGCAATGCTACGGTCCGAGCGCAGCAGTGA  
202 A A L V A H V T S R K C Y G P S A T S E

25 3121 AAAATGTCGCGGTAAACGCTCTGGAAAAAGCGGAAAAAGATCGATTACCGAACAGCTGCT  
222 K C P G N A L E K G G K G S I T E Q L L

3181 TAACGCTCGTCCGACGTTACGCTTGGCGGCGCGCAAAACCTTTGCTGAAACGGCAAC  
242 N A R A D V T L G G G A K T P A E T A T

30 3241 CGCTGCTGAATGGCAGGAAAAACGCTCGGTGAACAGGCACAGGCGCGTGGTTATCAGTT  
262 A G E W Q G K T L R E Q A Q A R G Y Q L

3301 GGTGAGCGATGCTGCCTCACTGAATTCGGTGACGGAAAGCGAATCAGCAAAAAACCCCTGCT  
35 282 V S D A A S L N S V T E A N Q Q K P L L

3361 TGGCCCTGTTTGTCTGACGGCAATATGCCAGTGGCGCTGGCTAGGACCGCAAGCAACGTACCA  
302 G L F A D G N M P V R W L G P K A T Y H

40 3421 TGGCAATATCGATAAGCCCGCAGTCACCTGTACGCCAAATCCGCCAACGTAATGACAGTGT  
322 G N I D K P A V T C T P N P Q R N D S V

3481 ACCAACCCCTGGCGCAGATGACCGACAAAGCCATTGAAATGTTGAGTAAAAATGAGAAAGG  
342 P T L A Q M T D K A I E L L S K N E K G

45 3541 CTTTTTCCTGCAAGTTGAAGGTGCGTCAATCGATAAACAGGATCACTGCTCGAATCCCTTG  
362 F F L Q V E G A S I D K Q D H A A N P C

3601 TGGCAAAATTTGGCGAGACGGTCGATCTCGATGAAGCCGTACAAACGGGCGCTGGAATTCGC  
50 382 G Q I G E T V D L D E A V Q R A L E F A

3661 TAAAAAGGAGGGTAACACGCTGCTCATAGTCACCGCTGATCAGCCCCACGCCACGCAGAT  
402 K K E G N T L V I V T A D H A H A S Q I

55 3721 TGTTCGCGCGGATACCAAGCTCCGGCCTCACCAGGCGCTAAATACCAAGATGGCGC  
422 V A P D T K A P G L T Q A L N T K D G A

3781 AGTGATGGTGATGAGTTACGGGAACCTCCGAAGAGGATTCAAGAACATACCGCGCAGTCA  
442 V M V M S Y G N S K E D S Q E H T G S Q

60

WO 03/072014

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3841 GTTGCCTATTGCGCGTATGCCCCGATGCCGCCAATGTTGTTGGACTGACCGACCAGAC  
462 L R I A A Y G P H A A N V V G L T D Q T

5 3901 CGATCTCTTCTACACCATGAAAGCGCTCTGGGGCTGAAATAATCTAGAGGATCCCCGGG  
482 D L F Y T M K A A L G L K

XbaI

10 SEQ ID NO 9

pMPX-33 (*toxR-AphaA* cloned into pMPX-5 using PCR-introduced PstI and XbaI)

15 2401 GAATTCCAGCGCTTTTITAGACTGGTGTATGAAATTCAGCAGGATCACATTCTGCAGAT  
Shine-Delgarno PstI  
M  
2461 GAACTTGGGGAATCGACTGTTTATTCTGATAGCGTCTTACTTCCCCTCGCAGTATTACT  
2 N L G N R L F I L I A V L P C L L A V L L  
20 2521 GCTCATGCGCTGTTCTGGAACCGGGCTGCTCAGGGCGATATTAAGTGCACCGCGGTGC  
22 L M P V L E N R A A Q G D I T A P G G A  
25 2581 TCGCGCTTTACGGGTGATCAGACTGCCGCTCTGCGTGATTCTCTTAGCGATAAACCTGC  
42 R R L T G D Q T A A L R D S L S D K P A  
2641 AAAAAATATTATTTGCTGATTGGCGATGGGATGGGGGACTCGGAAATTAAGTGCACG  
62 K N I I L L I G D G M G D S E I T A A R  
30 2701 TAATTATGCGAAGGTGCGGCGGCTTTTAAAGGTATAGTGCCTTACCGCTTACCGG  
82 N Y A E G A G G F F K G I D A L P L T G  
2761 GCAATACACTCATTATGCGCTGAATAAAAAACCGGCAACCGGACTACGTACCGACTC  
102 Q Y T H Y A L N K K T G K P D Y V T D S  
35 2821 GGCTGCATCAGCAACCGCTGTCACCGGTGTCACCACTATAACCGCGCTGGGCGT  
122 A A S A T A W S T G V K T Y N G A L G V  
2881 CGATATTACGAAAAAGATCACCAACGATTCTGGAATGGCAAAACCGGAGGTCTGGC  
142 D I H E K D H P T I L E M A K A A G L A  
2941 GACCGTAACTTTCTACCGCAGAGTTGACAGGATGCCACGCGCGTGGCTGGTGGACA  
162 T G N V S T A E L Q D A T P A A L V A H  
45 3001 TGTGACCTCGCGCAATGCTACGGTCCGAGCGCGACGAGTGAATAATGTCGGGTAAACGC  
182 V T S R K C Y G P S A T S E K C P G N A  
3061 TCTGGAAAAAGCGGAAAAGGATCGATTACCGAACAGCTGCTTAACGCTGTCGCGACGT  
202 L E K G G K G S I T E Q L L N A R A D V  
50 3121 TACGCTTGGCGCGCGCAAAACCTTTGCTGAACCGGCAACCGCTGGTGAATGGCAGGG  
222 T L G G G A K T F A E T A T A G E W Q G  
3181 AAAAAGCTGCTGTAACAGGCACAGCGCGTGGTTATCAGTTGGTGAGCGATGCTGCTC  
242 K T L R E Q A Q A R G Y Q L V S D A A S  
3241 ACTGAATTGGGTGACGGAAGCGAATCAGCAAAACCCCTGCTTGGCCTGTTTGTGACGG  
262 L N S V T E A N Q Q K P L L G L F A D G

WO 03/072014

PCT/US02/16877

3301 CAATATGCCAGTGGCTGGCTAGGACCGAAAGCAACGTACCATGGCAATATCGATAAGCC  
282 N M P V R W L G P K A T Y H G N I D K P

5 3361 CGCAGTCCACTGTACGCCAAATCCGCAACGTAAATGACAGTGTACCAACCTGGCGCAGAT  
302 A V T C T P N P Q R N D S V P T L A Q M

3421 GACCGACAAAGCCATTGAATTTGTAGTAAAAATGAGAAAGGCTTTTCTCTGCAAGTTGA  
322 T D K A I E L L S K N E K G F F L Q V E

10 3481 AGGTGCGTCAATCGATAAACAGGATCATGCTGCGAATCCTTGTGGGCAATTTGGCGAGAC  
342 G A S I D K Q D H A A N P C G Q I G E T

3541 GGTGCGATCTCGATGAAGCCGTACAACGGGCGCTGGAATTCGTAAAAAGGAGGCTAACAC  
362 V D L D E A V Q R A L E F A K K E G N T

15 3601 GCTGGTCAATAGTACCGCTGATCAAGCCACCGCAGCCAGATTGTTGCGCCGATACCAA  
382 L V I V T A D H A H A S Q I V A P D T K

3661 AGCTCGGGCCTCACCCAGGCGCTAAATACCAAGATGGCGCATGATGTTGATGAGTTA  
402 A P G L T Q A L N T K D G A V M V M S Y

3721 CGGGAAGCTCCGAAGAGGATTACAAAGAACATACCGGCAGTCAGTTGCGTATTGCGGGCGTA  
422 G N S E E D S Q E H T G S Q L R I A A Y

25 3781 TGGCCCGCATGCGCCCAATGTTGTTGGACTGACCGACAGACCGATCTCTTACACCAT  
442 G P H A A N V V G L T D Q T D L F Y T M

XbaI

3841 GAAAGCGCTCTGGGGCTGAAATAATAATCTAGAGGATCCCGGGTACCGAGCTCGAATT  
30 462 K A A L G L K

Non-bold, underlined sequence is *toxR* transmembrane domain segment that constitutes *toxR* residues 178-198. The remaining sequence is from *AphoA* constituting *phoA* residues 49-453.

35

SEQ ID NO 10

pMPX-7 expression vector

40

1 TCGCGCGTTT CGGTGATGAC GGTGAAAACC TGTGACACAT GCAGCTCCCG  
GAGACGGTCA  
61 CAGCTTGTCT GTAAGCGGAT GCGGGGAGCA GACAAGCCCC TCAGGGCGCG  
TCAGCGGGTG  
45 121 TTGGCGGGTG TCGGGGCTGG CTTAACATATG CGGCATCAGA GCAGATTGTA  
CTGAGAGTGC  
181 ACCATATGCG GTGTGAAATA CCGCACAGAT GCGTAAGGAG AAAATACCGC  
ATCAGCGCGC  
241 ATTCGCCATT CAGGCTGCGC AACTGTTGGG AAGGGCGATC GGTGCGGGCC  
50 TCTTCGCTAT  
301 TACGCCAGCT GGCGAAAGGG GGATGTGCTG CAAGGCGATT AAGTTGGGTA  
ACGCCAGGGT

55

HindIII

361 TTTCCAGTC ACGACGTGT AAAACGACGG CCAGTGCCAA GCTTCGCAGC  
GCTGTTCTTT

WO 03/072014

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421 TGCTCGCCTG CTGCGAGCTG GGTAAAGCGA CAAATTCTCA CCGTCTCCGG  
TGGTGGGGTA  
481 CAGGAGCTCA ATTAATACAC TAACGGACCG GTAAACAACC GTGCGTGTG  
TTTACCGGGA  
5 541 TAAACTCATC AACGTCTCTG CTAATAACT' GGCAGCCAAA TCACGGCTAT  
TGGTTAACCA  
601 ATTTTCAGAGT GAAAAGTATA CGAATAGAGT GTGCCCTTCG ACTATTCAAC  
AGCAATGATA

10 uidR Start  
661 GGCGCTCACC TGACAACGCG GTAAACTAGT TATTCAAGCT AACTATAATG  
GTTTAATGAT →

15 721 GGATAACATG CAGACTGAAG CACAACCGAC ACGGACCCGG ATCCTCAATG  
CTGCCAGAGA  
781 GATTTTITCA GAAAATGGAT TTCACAGTGC CTGATGAAA GCCATCTGTA  
AATCTTGGCG  
20 841 CATTAGTCCC GGAACGCTCT ATCACCAATT CATCTCCAAA GAAGCCTTGA  
TTCAGGCGAT  
901 TATCTTACAG GACCAGGAGA GGGCGCTGGC CCGTTTCCGG GAACCGATTG  
AAGGGATTCA  
961 TTTGGTTGAC TATATGGTCG AGTCCATTGT CTCTCTCACC CATGAAGCCT  
25 TTGACAAAG  
1021 GGCGCTGGTG GTTGAATAA TGCCGGAAGG GATGCGTAAC CCACAGGTG  
CCGCCATGCT  
1081 TAAAAATAAG CATATGACGA TCACGGAATT TGTTGCCAG CGGATGCGTG  
ATGCCAGCA  
30 1141 AAAAGGCGAG ATAAGCCAG ACATCAACAC GGCAATGACT TCACGTTTAC  
TGCTGGATCT  
1201 GACCTACGGT GTACTGGCCG ATATCGAAGC GGAAGACCTG GCGCGTGAAG  
CGTCGTTTGC

35 1261 TCAGGGATTA CGCGCGATGA TTGGCGGTAT CTTAACCGCA TCCTGATCTCT  
CTCTCTTTT Stop uidR

40 1321 CGGCGGGCTG GTGATAACTG TGCCCCGGT TCATATCGTA ATTTCTCTGT  
GCAAAAATTA  
1381 TCCTTCCCCG CTTCGGAGAA TTCCCCCAA AATATTCACT GTAGCCATAT  
GTCAATGAGG  
1441 TTTATCGTTC CCAATACGCT CGAACGAACG TTCGGTTGCT TATTTTATGG  
CTTCTGTCAA  
45 1501 CGCTGTTTTA AAGATTAAAT CGATCTATAT CACGCTGTGG GTATTGCAAT  
TTTTGGTTTT  
1561 TTGATCGCGG TGTCAGTTCT TTTTATTTC ATTTCTCTTC CATGGGTTTC  
TCACAGATAA  
1621 CTGTGTGCAA CACAGAATTG GTTAACTAAT CAGATTAAAG GTTGACCAGT  
50 ATTATTATCT

Shine-Delgarno PstI Sali XbaI KpnI  
1681 TAATGAGGAG TCCTGCAGGT CGACTCTAGA GGATCCCCGG GTACCGAGCT  
CGAATTCGTA

55 →

1741 ATCATGGTCA TAGCTGTTTC CTGTGTGAAA TTGTTATCCG CTCACAATTC  
CACACAACAT  
1801 ACGAGCCGGA AGCATAAAGT GTAAAGCCTG GGGTGCTTAA TGAGTGAGCT  
60 AACTCACATT

PCT/US02/16877

19/268

PCT/US02/16877

←

25 constitutes the protein to be expressed promoter region under the control of *uidR*.

50	421	TGGGGGCGCT	GTTCGCTAAG	TTTGGGTAT	TGTTGGCGCG	ACATGCGGAC
		ATATTTCGCC				
	481	AACGTGCTGT	AAAAACGACT	ACTTGAACGA	AAGCCTGCCG	TCAGGGCAAT
		ATCGAGAATA				
	541	CTTTTATCG	TATCGCTCAG	TACGCGCGCA	ACGTGGTTGA	TGCGCATCGC
55		GGTAAGTAGT				
	601	TGTTTTCATCG	TCAATTGCTAT	GACCCGCTGG	AATATCCCCA	TGCATAGT
		GGCGTTAAGT				

WO 03/072014

PCT/US02/16877

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661      TTGACGTGCT CAGCCACATC GTTGATGGTC AGCGCCTGAT CATAGTTTTC
GGCAATAAAG
721      CCCAGCATCT GGCTAACATA AAATTGCGCA TGGCGCGAGA CGCTGTTTTT
TGTGTGCGC
5 781      GAGGTTTTAT TGACCAGAAT CGGTTCCAG CCAGAGAGGC TAAATCGCTT
GAGCATCAGG
841      CCAATTTCAT CAATGGCGAG CTGGCGAATT TGCTGTTG GACTGTTTAA
TTCCTGCTGC
10 901      CAGCGGCGCA CTTCAAACGG GCTAAGTTGC TGTGTGCGCA GTGATTGAT
CACCATGCCG
961      TGAGTGACGT GGTTAATCAG GTCITTATCC AGCGCCAGG AGAGAAACAG
ATGCATCGGC
1021     AGATTAAAAA TCGCCATGCT CTGACAGGTT CCGGTATCTG TTAGTTGGTG
CGGTGTACAG
15 1081     GCCCAGAACA GCGTGATATG ACCCTGATTC ATATTCACTT TTTCAATTGT
GATCAGGTAT
1141     TCCACATCGC CATCGAAAGG CACATTCACT TCGACCTGAC CATGCCAGTG
GCTGGTGGGC
1201     ATGATATGCG GTGCGCGAAA CTCAACTCTC ATCCGCTGGT ATTCGGAATA
20 CAGCGACGCG

                                                                 Start melR
1261     GGGCTGCGGG TCTGTTTTTC GTGCTGCTG CACATAAAGC TATCTGTATT
25 CATGGATGGC ←

1321     TCTCTTTCCT GGAATATCAG AATTATGGCA GGAGTGAGGG AGGATGACTG
CGAGTGGGAG
1381     CACGGTTTTT ACCCTCTTCC CAGAGGGGCG AGGGGACTCT CCGAGTATCA
30 TGAGGCCGAA
1441     AACTCTGCTT TTCAGGTAAT TTATTCCCAT AAACCTCAGAT TTA CTGCTGC
TTCACGCAGG

                                                                 Shine-Delgarno PstI
35 1501     ATCTGAGTTT ATGGAATGC TCAACCTGGA AGCCGAAGGT TTTCTGCAGA
TTCGCTGCC

                                                                 SalI XbaI
40 BamHI
1561     ATGATGAAGT TATTCAAGCA AGCCAGGAGA TCTGGTACCC GGGTCGACTC
TAGAGGATCC

                                                                 KpnI
1621     CCGGGTACCG AGCTCGAATT CGTAATCATG GTCATAGCTG TTTCTGTGT
45 GAAATTGTTA
1681     TCCGCTCACA ATTCCACACA ACATACGAGC CGGAAGCATA AAGTGTAAAG
CCTGGGTGCG
1741     CTAATGAGTG AGCTAACTCA CATTAATTGC GTTGCGCTCA CTGCCCGCTT
TCCAGTCGGG
50 1801     AAACCTGTGC TGCCAGCTGC ATTAATGAAT CGGCCAACGC GCGGGGAGAG
GCGGTTTTCG
1861     TATTGGGCGC TCTTCCGCTT CCTCGCTCAC TGACTCGCTG CGCTCGGTGC
TTCGGCTGCG
1921     GCGAGCGGTA TCAGCTCACT CAAAGGCGGT AATACGGTTA TCCACAGAAT
55 CAGGGGATAA
1981     CGCAGGAAG AACATGTGAG CAAAGGCCA GCAAAGGCC AGGAACCGTA
AAAAGCCGC
2041     GTTGCTGGCG TTTTTCATA GGCTCCGCC CCCGTACGAG CATCACAAA
ATCGACGCTC

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WO 03/072014

PCT/US02/16877

2101 AAGTCAGAGG TGGCGAAACC CGACAGGACT ATAAGATAC CAGGCGTTTC  
 CCCCTGGAAG  
 2161 CTCCTCGTG CGCTCTCTG TTCGACCCCT GCCGCTTACC GGATACCTGT  
 CCGCCITTTCT  
 5 2221 CCCTTCGGGA AGCGTGGCGC TTTCTCATAG CTCACGCTGT AGGTATCTCA  
 GTTCGGTGTA  
 2281 GGTGTTTCGC TCCAAGCTGG GCTGTGTGCA CGAACCCCC GTTCAGCCCG  
 ACCGCTGCGC  
 2341 CTTATCCGGT AACTATCGTC TTGAGTCCAA CCCGTAAGA CACGACTTAT  
 CGCCACTGGC  
 10 2401 AGCAGCCACT GGTAAACAGGA TTACGACAGC GAGGTATGTA GGCCTGTCTA  
 CAGAGTCTTT  
 2461 GAAGTGGTGG CCTAACTACG GCTACACTAG AAGGACAGTA TTTGGTATCT  
 GCGCTCTGCT  
 15 2521 GAAGCCAGTT ACCTTCGGAA AAAGAGTTGG TAGCTCTTGA TCCGGCAAAAC  
 AAACCCCGC  
 2581 TGGTAGCGGT GGTITTTTTG TTTGCAAGCA CGACATTACG CGCAGAAAAA  
 AAGGATCTCA  
 2641 AGAAGATCCT TTGATCTTTT CTACGGGGTC TGACGCTCAG TGGAAACGAA  
 20 2701 AGGGATTTTG GTCATGAGAT TATCAAAAAG GATCTTCACC TAGATCCITT  
 TAAATTAAAA  
 Stop bla  
 25 2761 ATGAAGTTTT AAATCAATCT AAAGTATATA TGAGTAAACT TGGTCTGACA  
 GTTACCAATC  
 2821 CTTAATCAGT GAGGCACCTA TCTCAGCGAT CTGTCTATTT CGTTCATCCA  
 TAGTTGCCCTG  
 30 2881 ACTCCCCGTC GTGTAGATAA CTACGATACG GGAGGGCTTA CCATCTGGCC  
 CCAAGTGTGC  
 2941 AATGATACCG CGAGACCCAC GCTCACCAGC TCCAGATTTA TCAGCAATAA  
 ACCAGCCAGC  
 3001 CGGAAGSGCC GAGCGCAGAA GTGTCTCTGC AACTTTATCC GCCTCCATCC  
 35 AGTCTATTAA  
 3061 TTGTTGCCGG GAAGCTAGAG TAAGTAGTTC GCCAGTTAAT AGTTTGCSCA  
 ACGTTGTTGC  
 3121 CATTGCTACA GGCATCGTGG TGTCACGCTC GTCGTTTGGT ATGGCTTCAT  
 TCAGCTCCGG  
 40 3181 TTCCCAACGA TCAAGGCGAG TTACATGATC CCCCATGTTG TGCAAAAAAG  
 CGGTTAGCTC  
 3241 CTTCGGTCCT CCGATCGTGG TCAGAAGTAA GTTGCCCGCA GTGTTATCAC  
 TCATGGTTAT  
 3301 GGCAGCACTG CATAATCTC TTAAGTTCAT GCCATCCGTA AGATGCTTTT  
 CTGTGACTGG  
 45 3361 TGAGTACTCA ACCAAGTCAT TCTGAGAATA GTGTATCGCG CGACCGAGTT  
 GCTCTTGGCC  
 3421 GCGGTCATA CGGGATAATA CCGCGCCACA TAGCAGAACT TTAAGAGTGC  
 TCATCATTTGG  
 50 3481 AAAACGTTCT TCGGGCGGAA AACTCTCAAG GATCTTACCG CTGTTGAGAT  
 CCAGTTGAT  
 3541 GTAACCCACT CGTGACCCCA ACTGATCTTC AGCATCTTTT ACTTTCACCA  
 GCGTTTCTGG  
 3601 GTGAGCAAAA ACAGGAAGGC AAAATGCGCG AAAAAAGGGA ATAAGGCGCA  
 CACGGAAATG  
 55  
 Start bla  
 3661 TTGAATACTC ATACTCTTCC TTTTCAATA TTATTGAAGC ATTTATCAGG  
 GTTATTGTCT  
 60 ←

WO 03/072014

PCT/US02/16877

3721 CATGAGCGGA TACATATTTG AATGTATTTA GAAAAATAAA CAAATAGGGG  
TTCCGCGCAC  
3781 ATTTCCCGGA AAAGTGCCAC CTGACGTCTA AGAACCATT ATTATCATGA  
5 CATTACCTTA  
3841 TAAAAATAGG CGTATCACGA GGCCCTTTCCG TC

The segment *melR* through the *PmeI* control region was taken from the *E. coli* MG1655 chromosome using PCR-added *HindIII* and *PstI* restriction sites. This fragment was cut with *HindIII* and *PstI* and cloned into pUC-18 cut with the same enzymes. Italicized sequence constitutes both *melR* and protein to be expressed promoter region.

SEQ ID NO 12

15 pMPX-18 expression vector

1 TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG  
20 GAGACGGTCA  
61 CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG TCAGGGCGCG  
TCAGCGGGTG  
121 TTGGCGGGTG TCGGGGCTGG CTTAACATG CCGCATCAGA GCAGATTGTA  
CTGAGAGTGC  
25 181 ACCATATGCG GTGTGAAATA CCGCACAGAT GCGTAAGGAG AAAATACCGC  
ATCAGGCGCC  
241 ATTGCGCCATT CAGGCTGCGC AACTGTGTGG AAGGGCGATC GGTGCGGGCC  
TCTTCGCTAT  
301 TACGCCAGCT GCGGAAAGGG GGATGTGCTG CAAGGCGATT AAGTTGGGTA  
30 ACGCCAGGGT

361 TTCCAGTC ACGACGTGTG AAAACGACGG CCAGTGCCAA GCTTCAAGCC  
35 GTCAATTGTC

421 TGATTCGTTA CCAATTATGA CAACTTGAGC GCTACATCAT TCACTTTTC  
TTCACAACCG

40 481 GCACGGAAC CGTCGGGCT GGCCCCGGTG CATTTTFTTAA ATACCGCGCA  
GAAATAGAGT  
541 TGATCGTCAA AACCACATT GCGACCGAGC GTGGCGATAG GCATCCGGGT  
GGTGCTCAAA  
601 AGCAGCTTCG CCTGGCTGAT ACGTTGGTCC TCGCGCCAGC TTAAGACGCT  
45 AATCCCTAAC  
661 TGCTGGCGGA AAAGATGTGA CAGACGCGAC GGCGACAAGC AAACATGCTG  
TGCGACGCTG  
721 GCGATATCAA AATTGCTGTC TGCCAGGTGA TCGCTGATGT ACTGACAAGC  
CTCGGTACCC  
50 781 CGATTATCCA TCGGTGGATG GAGCGACTCG TTAATCGCTT CCATGCGCGC  
CAGTAACAAT  
841 TGCTCAAGCA GATTATCGC CAGCAGCTCC GAATAGCGCC CTTCCTCTTG  
CCCGCGTTA  
901 ATGATTTGCC CAAACAGGTC GCTGAAATGC GGCTGGTGCG CTTCATCCGG  
55 GCGAAAGAAC  
961 CCGTATTGG CAAATATTGA CGGCCAGTTA AGCCATTTCAT GCCAGTAGGC  
GCGCGACGA

WO 03/072014

PCT/US02/16877

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1021  AAGTAAACCC ACTGGTGATA CCATTCGCGA GCCTCCGGAT GACGACCGTA
GTGATGAATC
1081  TCTCCTGGCG GGRACAGCAA AATATCACCC GGTCCGCAAA CAAATTCTCG
TCCCTGATTT
5  1141  TTCACCACCC CCTGACCGCG AATGGTGAGA TTGAGAATAT AACCTTTCAT
TCCCRAGCGT
1201  CGGTGATATA AAAAATCGAG ATRACCGTTG GCCTCAATCG GCGTTAAACC
CGCCACCAGA
1261  TGGGCATTAA ACGAGTATCC CGGCAGCAGG GGATCATTTT GCGCTTCAGC
10  CATACTTTTC

                                Start araC
1321  ATACTCCCGC CATTCAGAGA AGAAACCAAT TGTCATATT GCATCAGACA
TTGCCGTCAC
15  ←

1391  TCGCTCTTTT ACTGGCTCTT CTCGCTAACC AAACCGGTAA CCCCCTTAT
TAAAAGCATT
1441  CTGTAACAAA GCGGGACCAA AGCCATGACA AAAACGCGTA AAAAAAGTGT
20  CTATAATCAC
1501  GGCAGAAAAG TCCACATTGA TTATTTGCAC GCGCTCACAC TTTGCTATGC
CATAGCATTT
1561  TTATCCATAA GATTAGCGGA TCCTACCTGA CGCTTTTTAT CGCAACTCTC
TACTGTTTCT
25

                                Shine-Delgarno      PstI      SalI      XbaI
1621  CCATACCCGT TTTTTTGGGC TAGCAGGAGG AATTCACCCT GCAGGTCGAC
TCTAGAGGAT
30  →

                                XmaI KpnI
1681  CCCCGGGTAC CGAGCTCGAA TTCGTAATCA TGGTCATAGC TGTTCCTGT
GTGAAATTGT
1741  TATCCGCTCA CAATTCCACA CAACATACGA GCCGGAAGCA TAAAGGTAA
AGCCTGGGGT
35  1801  GCCTAATGAG TGAGCTAACT CACATTAAIT GCGTTGCGCT CACTGCCCGC
TTTCCAGTCG
1861  GGAAACCTGT CGTGCCAGCT GCATTATGA ATCGCCCAAC GCGCGGGAG
AGCGCGTTTG
40  1921  CGTATTGGGC GCTCTTCGCG TTCTTCGCTC ACTGACTCGC TGCCTCGGT
CGTTCCGCTG
1981  CGGCGAGCGG TATCAGCTCA CTCAAAGGCG GTAATACCGT TATCCACAGA
ATCAGGGGAT
2041  AACGCAGGAA AGAACATGTG AGCAAAAGGC CAGCAAAAGG CCAGGAACCG
TAAAAGGCCC
45  2101  GCGTGTCTGG CGTTTTCCTA TAGGCTCCGC CCCCTGACG AGCATCACAA
AAATCGACGC
2161  TCAAGTCAGA GGTGGCGAAA CCCGACAGGA CTATAAAGAT ACCAGCGGTT
TCCCCCTGGA
2221  AGCTCCCTCG TGCGCTCTCC TGTTCGACCC CTGCGCTTAA CCGGATACCT
50  GTCCGCTTTT
2281  CTCCTTTCGG GAAGCGTGGC GCTTCTCAT AGCTCACGCT GTAGGTATCT
CAGTTGGGTG
2341  TAGGTCGTTT GCTCCAAGCT GGGCTGTGTG CACGAACCCC CCGTTCAGCC
CGACCGCTGC
55  2401  GCCTTATCCG GTAACATCG TCTTGAGTCC AACC CGGTAA GACACGACTT
ATCGCCACTG
2461  GCAGCAGCCA CTGTAACAG GATTAGCAGA GCGAGGTATG TAGGCGGTGC
TACAGAGTTC
2521  TTGAAGTGGT GGCTAACTA CGGCTACACT AGAAGGACAG TATTTGGTAT
60  CTGCGCTCTG

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PCT/US02/16877

55 The segment *araC* through the Para control region was taken from pBAD24 using PCR-added *Hind*III and *Pst*I restriction sites. This fragment was cut with *Hind*III and *Pst*I and cloned into pUC-18 cut with the same enzymes. Italicized sequence constitutes both *araC* and protein to be expressed promoter region.

WO 03/07214

PCT/US02/16877

SEQ ID NO 13

## 5 pMPX-6 expression vector

1           TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT AGCCCATATA  
 TGGAGTTCGG  
 10 61       CGTTACATAA CTTACGGTAA ATGGCCCCGCC TGGCTGACCG CCCAACGACC  
 CCGGCCCAATT  
 121       GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA GGGACTTTCC  
 ATTGACGTCA  
 181       ATGGGTGGAG TATTTACGGT AAATGCCCCA CTTGGCAGTA CATCAAGTGT  
 ATCATATGCC  
 15 241       AAGTAGCCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT  
 ATGCCCAGTA  
 301       CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA  
 TCGCTATTAC  
 20 361       CATGGTGATG CGGTTTTGGC AGTACATCAA TGGGCGTGGA TAGCGGTTTG  
 ACTCACGGGG  
 421       ATTTCCAAGT CTCACCCCCA TTGACGTCAA TGGGAGTTTG TTTTGGCACC  
 AAAATCAACG  
 481       GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG  
 GTAGGCGTGT  
 25 541       ACGGTGGGAG GTCTATATAA GCAGAGCTGG TTTAGTGAAC CGTCAGATCC  
 GCTAGCGCTA

Start GFP

30 601       CCGGTCGCCA CCATGCTGAG CAAGGGCGAG GAGCTGTTCA CCGGGTGTGT  
 GCCATCCTG

→

661       GTCGAGCTGG ACGGCGACGT AAACGGCCAC AAGTTCAGCG TGTCCGGCGA  
 GGGCGAGGGC  
 721       GATGCCACCT ACGGCAAGCT GACCCTGAAG TTCATCTGCA CCACCGGCAG  
 GCTGCCCGTG  
 781       CCCTGGCCCC CCCTCGTGAC CACCCTGACC TACGGCGTGC AGTGCTTCAG  
 CCGCTACCCC  
 841       GACCACATGA AGCAGCAGCA CTCTTCAAG TCCGCCATGC CCGAAGGCTA  
 CGTCCAGGAG  
 901       CGCACCATCT TCCTTCAAGGA CGACGGCAAC TACAAGACCC GCGCCGAGGT  
 GAAGTTCGAG  
 961       GGCGACACCC TGGTGAACCG CATCGAGCTG AAGGGCATCG ACTTCAAGGA  
 GGACGGCAAC  
 1021       ATCCTGGGGC ACAAGCTGGA GTACAACCTAC AACAGCCACA ACGTCTATAT  
 CATGGCCGAC  
 1081       AAGCAGAAGA ACGGCATCAA GGTGAACCTC AAGATCCGCC ACAACATCGA  
 GGAACGGCAGC  
 1141       GTGCAGCTCG CCGACCACTA CCAGCAGAAC ACCCCCATCG GCGACGGCCC  
 CGTGCTGCTG  
 1201       CCCGACAACC ACTACCTGAG CACCAGTCC GCCCTGAGCA AAGACCCCAA  
 CGAGAACGCG  
 1261       GATCACATGG TCCTGCTGGA GTTCGTGACC GCCGCCGGGA TCATCTCTCGG  
 CATGACGAG

XhoI Stop GFP

1321       CTGTACAAGT CCGGACTCAG ATCTCGAGCT TAATAACAAG CCGTCAATTG  
 TCTGATTCTG

PCT/US02/16877

27/268

PCT/US02/16877

Start Kan

→

28/268

WO 03/072014

PCT/US02/16877

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4501  TGGAAAATGG  CCGCTTTTCT  GGATTCATCG  ACTGTGGCCG  GCTGGGTGTG
GCGGACCGCT
4561  ATCAGGACAT  AGCGTTGGCT  ACCCGTGATA  TTGCTGAAGA  GCTTGGCGGC
GAATGGGCTG
5  4621  ACCGCTTCCT  CGTGCTTTAC  GGTATCGCCG  CTCCCATTTC  GCAGCGCATC
GCCTTCTATC

                                Stop Kan
4681  GCCTTCTTGA  CGAGTTCTTC  TGAGCGGGAC  TCTGGGGTTC  GAAATGACCG
10  ACCAAGCGAC

4741  GCCCAACCTG  CCATCACGAG  ATTTGCGATT  CACCGCCGCC  TTCTATGAAA
GGTTGGGCTT
4801  CGGAATCGTT  TTCCGGGACG  CCGGCTGGAT  GATCCTCCAG  CGCGGGGATC
15  TCATGCTGGA
4861  GTTCTTCGCC  CACCCTAGGG  GGAGGCTAAC  TGAAACACGG  AAGGAGACAA
TACCGGAAGG
4921  AACCCGCGCT  ATGACGGCAA  TAAAAAGACA  GAATAAAACG  CACGGTGTTC
GTCGTTTGT
20  4981  TCATAAACGC  GGGGTTGCGT  CCCAGGGCTG  GCACCTGTCT  GATACCCAC
CGAGACCCCA
5041  TTGGGGCCAA  TACGCCCGCG  TTTCTTCTTT  TTCCCCACCC  CACCCCCCAA
GTTCCGGTGA
5101  AGGCCCAGGG  CTCGAGCCA  ACGTCGGGGC  GGCAGGCCCT  GCCATAGCCT
25  CAGGTTACTC
5161  ATATATACTT  TAGATTGATT  TAAAACTTCA  TTTTTAATTT  AAAAGGATCT
AGGTGAAGAT
5221  CCTTTTGTAT  AATCTCATGA  CAAAATATCC  TTAACGTGAG  TTTTCGTTCC
ACTGAGCGTC
30  5281  AGACCCCGTA  GAAAAGATCA  AAGGATCTTC  TTGAGATCCT  TTTTTCGTGC
CGTAATCTG
5341  CTGCTTGCAA  ACAAAAAAAC  CACCGCTACC  AGCGGTGGTT  TGTTCGCCGG
ATCAAGAGCT
5401  ACCAACTCTT  TTCCGGAAGG  TAACTGGCTT  CAGCAGAGCG  CAGATACCAA
35  ATACTGTCTC
5461  TCTAGTGTAG  CCGTAGTTAG  GCCACCACTT  CAAGAACTCT  GTAGCACCGC
CTACATACTT
5521  CGCTCTGCTA  ATCTGTGTAC  CAGTGGCTGC  TGCCAGTGGC  GATAAGTCGT
GTCCTACCGG
40  5581  GTTGGACTCA  AGACGATAGT  TACCGGATAA  GCGCGCAGCG  TCGGGCTGAA
CGGGGGGTTT
5641  GTGCACACAG  CCCAGCTTGG  AGCGAACGAC  CTACACCGAA  CTGAGATACC
TACAGCGTGA
5701  GCTATGAGAA  AGCGCCACGC  TTCCGAAGG  GAGAAAGCG  GACAGGTATC
45  CGGTAAGCGG
5761  CAGGGTCGGA  ACAGGAGAGC  GCACGAGGGA  GCTTCCAGGG  GGAAACGCTT
GGTATCTTTA
5821  TAGTCTCTGC  GGGTTTCGCC  ACCTCTGACT  TGAGGCTCGA  TTTTGTGAT
GCTCGTCAGG
50  5881  GGGGCGGAGC  CTATGGAAAA  ACGCCAGCAA  CGCGGCCTTT  TTACGGTTCC
TGGCCTTTTC
5941  CTGGCCCTTT  GCTCATATGT  TCTTCTCTGC  GTTATCCCTT  GATTCTGTGG
ATAACCGTAT
6001  TACCGCCATG  CAT
55

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The segment *araC* through *SsrII* following the Para control region was taken from pBAD24 using a PCR-added *XhoI* restriction site. This fragment was cut with *XhoI* and *SsrII* and cloned into pEGFP-C1 (Clontech) cut with the same enzymes. Italicized and underlined

WO 03/072014

PCT/US02/16877

sequence constitutes the CMV promotor region while the italicized alone region constitutes both the *araC* and protein to be expressed promotor region.

5

SEQ ID NO 14

10

pMPX-56 (rat Edg3 cloned into pMPX-5 using PCR-introduced SalI and KpnI)

15	2401	GAATT	CAGCGCTTTT	TAGACTGGT	CGTAATGAA	ATTCAGCAGGATCACATCTTG	CAGGT
							Shine-Delgarno
							SalI
	2461	CGACA	TGGCAACCA	CGCAGCGC	CAGGGCCACCCG	CAGCTCTGGGGAATG	ATACTCTCCG
20	1		M A T T H A Q G H P P V L G N D T L R				
	2521	GGAAC	ATTATGATTAC	GTGGGGAAGCTGGCAGGCAGGCTGCGGGATCCCCCTGAGGGTAG			
	20		E H Y D Y V G K L A G R L R D P P E G S				
	2581	CACCC	TCATCACCA	CCATCCTCTTCTTGGTCACTGTAGCTTCA	TCGTCCTTGGAGA	ACCT	
25	40		T L I T T I L F L V T C S F I V L E N L				
	2641	GATGG	TTTGTGATGCCATCTGG	AAAAACAATAAATTTCA	TAAACCGCATGTACTTTTTCAT		
	60		M V L I A I W K N N K F H N R M Y F P F I				
30	2701	CGGCA	ACTTGGCTCTCTGCGACCTGCTGGCCGCGCATAGCCTACAAGGTCAACATTCTGAT				
	80		G N L A L C D L L A G I A Y K V N I L M				
	2761	GTCGG	TAGGAAGA	CGTTTCA	GCCTGTCTCCAACAGTGTGGTTTCTC	CAGGGAGGGCAGTAT	
	100		S G R K T F S L S P T V W F L R E G S M				
35	2821	GTTCT	GAGCCCTGGGCGCATCC	CATGCAGCTTATTGGCCATTG	CCATTGAGCGGCACCT		
	120		F V A L G A S T C S L L A I A I E R H L				
	2881	GACCAT	GTATCAAGATGAGGCCGTAC	GACGCCAACAGAACACCGCGTGT	TCTCTCTGAT		
40	140		T M I K M R P Y D A N K K H R V F L L I				
	2941	TGGGA	TGTGCTGGCTAATTG	CCTTCTCGCTGGGTGCCCTGCCCATCCTGGGCTGGA	ACTG		
	160		G M C W L I A F S L G A L P I L G W N C				
45	3001	CCTG	AAAACTTTCCGACTGCTCTACCATCTT	GGCCCTCTACTCCAGAAATACATTGC			
	180		L E N F P D C S T I L P L Y S K K Y I A				
	3061	CTTCT	CATCAGCATCTTCATAGCCATTCTGGTGACCATCGTCATCTTGTACGCGCGCAT				
50	200		F L I S I F I A I L V T I V I L Y A R I				
	3121	CTACT	TCTGTGTAAGTCCAGCAGCCG	CAGGGTGGCCAAACCAACTCCGAGAGATCCAT			
	220		Y F L V K S S S R R V A N H N S E R S M				
	3181	GGCC	CTTCTGCGGACGTAGTGATCGTGGTGAGCGTGTT	CATCGCCTTGTGGTCCCCCTC			
55	240		L L R T V V I V S V F I A C W S P L				
	3241	TTTCAT	CCTCTTCTCATCGATGTGGCCTG	CAGGGCGAAGGAGTGCTCCATCCTCTTCAA			
	260		F I L F L I D V A C R A K E C S I L F K				

PCT/US02/16877

pMPX-57 ( $\beta 2$  Adrenergic receptor ( $\beta 2AR$ ) cloned into pMPX-5 using PCR-introduced SalI and BamHI)

Shine-Delgarno

SalI

CGCATG GGGCAACCCGGGAACGGCAGCGCCTTCTTGCTGGCACCCAATGGAAGCCATGC  
M G O P G N G S A F L L A P N G S H A

GCCGGACCACGACGTCACGCAGCAAAGGGACGAGGTGTGGGTGGTGGGCATGGGCATCGT  
P D H D V T O O R D E V W V V G M G I V

CATGTCTCTCATCGTCCTGGCCATCGTGTTTGGCAATGTGCTGGTCATCACAGCCATTGC  
M S L I V L A I V F G N V L V I T A I A

CAAGTTCGAGCGTCTGCAGACGGTCACCAACTACTTCATCACTTCACTGGCCTGTGCTGA  
K F E R L O T V T N Y F I T S L A C A D

TCTGGTCATGGGCCCTAGCAGTGGTGCCTTTGGGGCCGCCCATATTCTTATGAAAATGTG  
L V M G L A V V P F G A A H I L M K M W

GACTTTTGGCAACTTCTGGTGCAGTTTTGGACTTCCATTGATGTGCTGTGCGTCACGGC  
T F G N F W C E F W T S I D V L C V T A

CAGCATTGAGACCTGTGCGTGATCGCAGTGGATCGCTACTTTGCCATTACTTCACCTTT  
S I E T L C V I A V D R Y F A I T S P F

PCT/US02/16877

45

AATTGGTACC TCAATGATGA TGATGATGAT GCTTGCAGAG GACCCCATTC TG

SEO ID NO 17

55

Shine-Delgarno

TCGCAACTCTCTACTGTTTCTCCATACCCGTTTTTTTGGGCTAGCAGGAGGAATTCA CCA

WO 03/072014

PCT/US02/16877

NcoI  
1321 TGGTAGTGTGTGTCCTCCCAAGGAAAATATATCCACCCTCAAATAATTCGATTGTGTGA  
1 M D S V C P Q G K Y I H P Q N N S I C C  
5  
1381 CCAAGTCCACAAAGGAACCTACTTGTACAATGACTGTCCAGGCCCGGGGAGGATACGG  
21 T K C H K G T Y L Y N D C P G P G Q D T  
10  
1441 ACTGCAGGGAGTGTGAGAGCGGCTCCTTCACCGCTTCAGAAAACCACTCAGACACTGCC  
41 D C R E C E S G S F T A S E N H L R H C  
1501 TCAGTGTCTCCAATGCCGAAAGGAAATGGGTGAGTGGAGATCTCTTCCTGCACAGTGG  
61 L S C S K C R K E M G Q V E I S S C T V  
15  
1561 ACCGGGACACCGTGTGTGGCTGCAGGAAGAACCACTACCGGCATTATTGGAGTGAAGAAC  
81 D R D T V C G C R K N Q Y R H Y W S E N  
1621 TTTTCCAGTGTCTCAATTGCGAGCCTTCGCTCAATGGGACCGTGCACCTCTCTCTGCCAGG  
101 L F Q C F N C S L C L N G T V H L S C C Q  
20  
1681 AGAAACAGAAACACCGTGTGCACCTGCACGAGTTTCTTCTTAAGAGAAAACGAGTGTG  
121 E K Q N T V C T C H A G F F L R E N E C  
1741 TCTCTGTAGTAACCTGTAAGAAAAGCTGGAGTGCACGAAGTTGTGCTACCCAGATTG  
141 V S C S N C K K S L E C T K L C L P Q I  
25  
1801 AGAATGTTAAGGGCACTGAGGACTCAGGCACCAAGTGTGTGTGCCCTGGTCACTTTTCT  
161 E N V K G T E D S G T T V L L P L V I F  
30  
1861 TTGGTCTTTGGCTTTTATCCCTCCTCTTCATTGGTTTAAATGTATCGTACCAACGGTGA  
181 F G L C L L S L L F I G L M F Y R Y Q R W  
1921 AGTCCAAGCTCTACTCCATTGTTTGTGGGAAATCGACACCTGAAAAAGAGGGGAGCTTG  
201 K S K L Y S I V C G K S T P E K E G E L  
35  
1981 AAGGAAGCTACTTAAGCCCTGGCCCAACCAAGCTTCAGTCCCACTCCAGGCTTCA  
221 E G T T T K P L A P N P S F S P T P G F  
2041 CCCCCCCTGGGCTTCAGTCCCGTGGCCCACTTCCACCTTCACCTCCAGCTCCACCTATA  
241 T P T L G F S P V P S T T T S S S T Y  
2101 CCCCCGTGACTGTCCCAACTTTTGGGCTCCCCGACAGAGGGTGGCACCACCTATCAGG  
261 T P G D C P N F A A P R R E V A P P Y Q  
45  
2161 GGGCTGACCCCACTCTTGCAGACGCCCTCGCTTCGACCCCATCCCAACCCCTTCAGA  
281 G A D P I L A T A L A S D P I P N P L Q  
2221 AGTGGGAGGACAGCGCCCAAGCCACAGAGCCTAGACACTGATGACCCCGGAGCGCTGT  
301 K W E D S A H K P Q S L D T D P A D T L  
50  
2281 ACGCGTGGTGGAGAAGTGCCTCCCGTGGCGCTGGAGGGAATTCGTGCGCGCCCTAGGGC  
321 Y A V V E N V P P L R W K E F V R R L G  
2341 TGAGCGACCAAGAGATCGATCGGCTGGAGCTGCAGAACGGGCGCTGCCTGCGCGAGCGGC  
341 L S D H E I D R L E L Q N G Y R C L E A  
55  
2401 AATACAGCATGCTGGCGACCTGGAGGCGGCGCACGCGCGCGAGGGCACTGAGAC  
361 Q Y S M L A T W R R R T P R R E A T L E  
60  
2461 TGCTGGGACGCGTGTCTCCGACATGGACCTGCTGGGCTCGCTGGAGGACATCGAGGAGG

WO 03/072014

PCT/US02/16877

381 L L G R V L R D M D L L G C L E D I E E

2521 CGCTTTGGCGCCCGCCGCTCCCGCCGCGCCAGTCTTCTCAGATGATCTAGAGTCCG XbaI

5 401 A L C G P A A L P P A P S L L R

10

SEQ ID NO 18

15 pMPX-22 (Human tumor necrosis factor receptor (TNFR-1) residues 29-455 cloned into pMPX-18 using PCR-introduced SalI and KpnI)

20 1621 CCATACCCGTTTTTTTGGGCTAGCAGGAGGAATTCACCTGCAGTCCGACATGGGACTGG Shine-Delgarno SalI  
1 M G L

1681 TCCCTCACCTAGGGGACAGGAGAGAGATAGTGTGTGTCCCCAAGGAAATATATCC  
4 V P H L G D R E K R D S V C P Q G K Y I

25 1741 ACCCTCAAAATAATTGCTGTACCAAGTGCCACAAGGAACCTACTTGTACAATG  
24 H P Q N N S I C C T K C H K G T Y L Y N

1801 ACTGTCCAGGCCCGGGCAGGATACGGAAGTGTGAGAGCGGCTCCTTCACCG  
30 44 D C P G P G Q D T D C R E C E S G S F T

1861 CTTCAGAAAACCACTCAGACACTGCCTCAGCTGCTCCAAATGCCGAAAGAAAATGGGTC  
64 A S E N H L R H C L S C S K C R K E M G

35 1921 AGGTGGAGATCTCTCTTGCACAGTGGACCGGACACCGTGTGTGGCTGCAGGAAGAACC  
84 Q V E I S S C T V D R D T V C G C R K N

1981 AGTACCGGCATTATTGGAGTGAAAACCTTTCCAGTGCTTCAATTGCAGCCTCTGCTCA  
104 Q Y R H Y W S E N L F Q C F N C S L C L

40 2041 ATGGGACCGTGCACTCTCCTGCCAGAGAAACAGAAACCGGTGTGCACTGCCATCAG  
124 N G T V H L S C Q E K Q N T V C T C H A

2101 GTTCTTTCTAAGAGAAAACGAGTGTGTCTCCTGTAGTAACGTAAAGAAAAGCCTGGAGT  
45 144 G F F L R E N E C V S C S N C K K S L E

2161 GCACGAAGTGTGCCTACCCGAGATTGAGAATGTTAAGGGCACTGAGGACTCAGGCACCA  
164 C T K L C L P Q I E N V K G T E D S G T

50 2221 CAGTGTGTGTGCCCTGGTCATTTCTTTGTCTTTGCCCTTTTATCCCTCCTCTTCATTG  
184 T V L L P L V I F F G L C L L S L L P I

2281 GTTTAATGTATCGCTACCAACGGTGGAGTCCAAGCTCTACTCCAATTGTTTGTGGGAAAT  
204 G L M Y R Y Q R W K S K L Y S I V C G K

55 2341 CGACACCTGAAAAAGAGGGGAGCTTGAAGGAACCTACTACTAAGCCCTGGCCCCAAACC  
224 S T P E K E G E L E G T T T K P L A P N

WO 03/072014

PCT/US02/16877

2401 CAAGCTTCAGTCCCACTCCAGGCTTCACCCCAACCTGGGCTTCAGTCCCGTCCCAAGTT  
 244 P S F S P T P G F T P T L G F S P V P S  
 2461 CCACCTTCACCTCCAGCTCCACCTATACCCCGGTGACTGCCAACTTTGCGGCTCCCC  
 5 264 S T F T S S S T Y T P G D C P N F A A P  
 2521 GCAGAGAGGTGGCAACCACTTATCAGGGGGCTGACCCCATCTTTCGACAGCCCTCGCCT  
 284 R R E V A P P Y Q G A D P I L A T A C A L A  
 10 2581 CCGACCCCATCCCCAACCCCTTCAGAAAGTGGGAGGACAGCGCCCAACAGCCACAGAGCC  
 304 S D P I P N P L Q K W E D S A H K P Q S  
 2641 TAGACACTGATGACCCCGGACGCTGTACGCCGTGGTGGAGAACGTGCCCCCGTTGCGCT  
 15 324 L D T D D P A T L Y A V V E N V P P L R  
 2701 GGAAGGAATTCGTGCGGCGCCTAGGGCTGAGCGACACAGAGATCGATCGGCTGGAGCTGC  
 344 W K E F V R R L G L S D H E I D R L E L  
 20 2761 AGAACGGGCGCTGCCTGCGCGAGGCGCAATACAGCATGCTGGCGACCTGGAGGCGGCGCA  
 364 Q N G R C L R E A Q Y S M L A T W R R R  
 2821 CGCCGCGGCGCGAGGCCACGCTGGAGCTGCTGGGACGCTGCTCCGCGACATGGACCTGC  
 384 T P R R E A T L E L L G R V L R D M D L  
 25 2881 TGGGCTGCTGGAGGACATCGAGGAGGCGCTTTGCGGCCCCGCCGCCCTCCGCCCGCGC  
 404 L G C L E D I E E A L C G P A A L P F A  
 KpnI  
 2941 CCAGTCTTCTCAGATAATAAGGTACCGAGCTCGAATTCGTAATCATGGTCATAGCTGTTT  
 30 424 P S L L R

35

SEQ ID NO 19

40 pMPX-40 (Human tumor necrosis factor (TNF) cloned into pMPX-6 using PCR-introduced  
 EcoRI and HindIII)

EcoRI  
 Shine-Delgarno  
 2581 GTTTTTTTGGGCTAGCAGGAGGAATTCATGAGCACTGAAAGCATGATCCGGGACGTGGAG  
 1 M S T E S M I R D V E  
 2641 CTGGCCGAGGAGGCGCTCCCCAAGAAGACAGGGGGGCCAGGGCTCCAGGCGGTGCTTG  
 12 L A E E A L P K K T G G P Q G S R R C L  
 2701 TTCTCAGCCTCTTCTCTCTCTGATCGTGGCAGGCGCCACCAAGCTCTTCTGCTGCTG  
 15 32 F L S L F S F L I V A G A T T L F C L L  
 2761 CACTTTGGAGTGATCGGCCCCAGAGGAAGAGTTCCCCAGGGACCTCTCTTAATCAGC  
 52 H F G V I G P Q R E E F P R D L S L I S  
 2821 CCTCTGGCCAGGCACTCAGATCATCTTCTCGAACCCGAGTGACAGGCTGTAGCCCAT  
 55 72 P L A Q A V R S S S R T P S D K P V A H  
 2881 GTTGTAGCAACCCCTCAAGCTGAGGGGCGAGCTCAGTGGCTGAACCCGCGGCCAATGCC  
 92 V V A N P Q A E G Q L Q W L N R R A N A

WO 03/072014

PCT/US02/16877

2941 CTCCTGGCCAATGGCGTGGAGCTGAGAGATAACAGCTGGTGGTGCCATCAGAGGGCCTG  
112 L L A N G V E L R D N Q L V V P S E G L

5 3001 TACCTCATCTACTCCAGGTCTCTTTCAAGGGCAAGGCTGCCCTCCACCCATGTGCTC  
132 Y L I Y S Q V L F K G Q G C P S T H V L

3061 CTCACCCACACCATCAGCGCATCGCCGCTCTCTACCAGACCAAGGTCAACCTCTCTCT  
152 L T H T I S R I A V S Y Q T K V N L L S

10 3121 GCCATCAAGAGCCCTGCCAGAGGGAGACCCAGAGGGGGCTGAGGCCAAGCCCTGGTAT  
172 A I K S P C Q R E T P E G A E A K P W Y

3181 GAGCCCATCTATCTGGGAGGGTCTTCCAGCTGGAGCAAGGGTGACCGACTCAGCGCTGAG  
192 E P I Y L G G V F Q L E K G D R L S A E

15 3241 ATCAATCGGCCCGACTATCTCGACTTTGCCGAGTCTGGGCAAGTCTACTTTGGGATCATT  
212 I N R P D Y L D F A E S G Q V Y F G I I

20 HindIII  
3301 GCCCTGTGATAAGCTTGGCCCGCGGGCCCGGGATCCACCGGATCTAGATAACTGATCATA  
232 A L

25

SEQ ID NO 20

30 pMPX-52 (*toxR*-EGF cloned into pMPX-6 using PCR-introduced KpnI and HindIII)

Shine-Delgarno KpnI  
2581 GTTTTTTTGGGCTAGCAGGAGGAATTCACCATGGTACCATGAACCTGGGGAAATCGACTGT  
1 M N L G N R L

35 2641 TTATTCTGATAGCGGTCTTACTTCCCTCGCAGTATTACTTGCTCAATAGTGACTCTGAAT  
8 F I L I A V L L P L A V L L L N S D S E

2701 GTCCCTGTCCACGATGGGTACTGCCTCCATGATGGTGTGTGCATGTATATGAAGCAT  
40 28 C P L S H D G Y C L H D G V C M Y I E A

2761 TGGACAAGTATGCATGCAACTGTGTGTGGCTACATCGGGGAGCGATGTCACTACCGAG  
48 L D K Y A C N C V G Y I G E R C Q Y R

45 HindIII  
2821 ACCTGAAGTGCTGGGAACTGCGCTAATAAGCTTGGCCCGCGGGCCCGGATCCACCGGAT  
68 D L K W W E L R

50 Non-bold, underlined sequence is *toxR* transmembrane domain segment that constitutes *toxR* residues 178-198. The remaining sequence is from human EGF constituting EGF residues 971-1023.

55

SEQ ID NO 21

WO 03/072014

PCT/US02/16877

pMPX-27 (*toxR*-invasin cloned into pMPX-6 using PCR-introduced *EcoRI* and *PstI*)

**EcoRI**  
**Shine-Delgarno**

5 2581 GTTTT TTTGGGCTAGCAGGAGCAATTACCATGAACCTGGGGAATCGACTGTTTATCTCTG  
1 M N L G N R L F I L

2641 ATAGCGGTCTTACTTCCCCTCGCAGTATTACTGCTCTCATTACATAGGACGTCACCGTT  
10 11 I A V L L L P L A V L L L S F T L S V T V

2701 CAGCAGCCTCAGTTGACATTAACGGCGGCCGTCAITGGTGATGGCGACCGGCTAATGGG  
31 Q Q P Q L T L T A A V I G D G A P A N G

2761 AAAACTGCAATCACCGTTGAGTTTACCGTTGCTGATTTTGGGGGAAACCCTTAGCCGGG  
15 51 K T A I T V E F T V A D F E G K P L A G

2821 CAGGAGGTGGTATAACCAACAATAATGGTGGCTACCGAATAAAATCAGGAAAGACA  
20 71 Q E V V I T T N N G A L P N K I T E K T

2881 GATGCAAAATGGCGTGGCGGCATTGCAATTAACCAATACGACAGATGGCGTGACGGTAGTC  
91 D A N G V A R I A L T N T T D G V T V V

2941 ACAGCAGAAGTGGAGGGGGCAACGGCAAGTGTTGATACCCACTTTGTTAAGGGTACTATC  
25 111 T A E V E G Q R Q S V D T H F V K G T I

3001 GCGGCGGATAAAATCCACTCTGGGTGCGGTACCGACATCATCGCTGATGGTCTAATG  
131 A A D K S T L A A V P T S I I A D G T M

3061 GCTTCAACCATCAGCTTGGAGTTGAAGGATACCTATGGGGACCCGACGGCTGGCGCAAT  
30 151 A S T I T L E L K D T Y G D P Q A G A N

3121 GTGGCTTTTGACACAACCTTAGGCAATATGGGCGTTATCACGGATCAATGACGGCACT  
171 V A F D T T L G N M G V I T D H N D G T

3181 TATAGCGCACCATTTGACAGTACCAACGTTGGGGGTAGCAACAGTAACGGTGAAAGTGGAT  
35 191 Y S A P L T S T T L G V A T V T V K V D

3241 GGGGCTGCGTTCAGTGTGCCGAGTGTGACGGTTAATTTACCGGCAGATCCTATTCCAGAT  
40 211 G A A F S V P S V T V N F T A D P I P D

3301 GCTGGCGCGTCCAGTTTCAACCGTCTCCACACCGGATATCTGGCTGATGGCAGATGAGT  
231 A G R S S F T V S T P D I L A D G T M S

3361 TCCACATTATCCITGTCCCTGTGATAAGAATGGCCATTTTTACAGTGGGATGCAGGGC  
45 251 S T L S F V P V D K N G H F I S G M Q G

3421 TTGAGTTTACTCAAACCGTGTGCCGGTGAGTATTAGCCCCATTAACGAGCAGCCAGAT  
271 L S F T Q N G V P V S I S P I T E Q P D

3481 AGCTATACCGCGACGGTGGTTGGGAATAGTGTGGTGATGTCAATCACGCCCGCAGGTT  
50 291 S Y T A T V V G N S V G D I T I T P Q V

3541 GATACCTGATACTGAGTACATTGCAGAAAAAATATCCCTATTTCCCGGTACCTACGCTG  
55 311 D T L I L S T L Q K K I S L F P V P T L

3601 ACCGGTATTCTGGTTAACGGGGCAAAATTTTCGTACGGATAAAGGGTTCCCGAAAACGATC  
331 T G I L V N G Q N F A T D K G F P K T I

WO 03/072014

PCT/US02/16877

3661 TTATAAAACGCCACATTCACAGTTACAGATGGATAACGATGTTGCTAATAACTCAGTAT  
351 F K N A T F Q L Q M D N D V A N N T Q Y

3721 GAGTGGTTCGTCTCATTACACACCCAAATGTATCGGTAAACGATCAGGGTCAGGTGACGATT  
5 371 E W S S S F T P N V S V N D Q G Q V T I

3781 ACCTACCAACCTATAGCGAAGTGGCTGTGACGGCGAAAAGTAAAAAATCCCAAGTTAT  
391 T Y Q T Y S E V A V T A K S K K F P S Y

3841 TCGGTGAGTTATCGGTTCACCCAAATCGGTGGATATACGATGGCGGCAGATCGCTGGTA  
10 411 S V S Y R F Y P N R W I Y D G G R S L V

3901 TCCAGTCTCGAGGCCACGACACAATGCCAAGGTCAGATATGTCTGCGGTTCTTGAATCC  
431 S S L E A S R Q C Q G S D M S A V L E S

3961 TCACGTGCAACCAACGGAAACGCGTGCCTGACGGGACATTGTGGGGCGAGTGGGGGAGC  
15 451 S R A T N G T R A P D G T L W G E W G S

4021 TTGACCGCGTATAGTTCTGATTGGCAAATCTGGTGAATATTGGGTCAAAAAGACCAGCAG  
20 471 L T A Y S S D W Q S G E Y W V K K T S T

4081 GATTTTGAACCATGAATATGGACACAGGCGCACTGCACACAGGCGCTGCATACCTTGGCG  
491 D F E T M' N M D T G A L Q P G P A Y L A

25 4141 TTCCCGCTCTGTGCGCTGTCAATATAACTGCAGSCATGCAAGCTTGGCCCGGGCCCCGG  
511 P P L C A L S I PstI

30 Non-bold, underlined sequence is *toxR* transmembrane domain segment that constitutes *toxR* residues 178-198. The remaining sequence is from *Yersinia pseudotuberculosis* *inv* constituting *inv* residues 490-986.

35 SEQ ID NO 22

pMPX-59 (*phoA* leader cloned into pMPX-5 using PCR-introduced PstI and XbaI)

40 2401 GAATTTCAGGCGCTTTTCTAGACTGGTCGTAATGAAATTCAGCAGGATACACATTCTGCGAGAT  
Shine-Delgarno PstI  
M

2461 GTTCACGGCCGAGACTTATAGTCGCTTTGTTTATTTTATTTTAAATGTTATTGTACATGGAGA  
2 S R P R L I V A L F L F F N V F V H G E

45 2521 AAATAAAGTGAACAAAGCACTATTGCACTGGCACTCTTACCGTTACTGTTACCCCTGT  
22 N K V K Q S T I A L A L L P L L F T P V

50 2581 GACAAAAGCCCGGACACAGAATCTAGA  
42 T K A R T P E S R XbaI

55 *PhoA* leader (residues 1-48) from *E. coli* MG1655 cloned into pMPX-5. Create chimeric fusions with the *phoA* leader by cloning into XbaI and introducing a stop sequence.

SEQ ID NO 23

Shine-Delgarno Psd

2401 GAATTCAGGCGCTTTTGTAGACTGGTCGTAATGAAATTCAGCAGGATCACCATTCTGCAGAT  
1 M

2461 GTCACGCCGAGACTTATAGTCGCTTTGTTTTATTTTTTAATGTATTGTACATGGAGA  
2 S R P R L I V A L F L F F N V F V H G E

2521 AAATAAAGTGAACAAAGCACTATTGCACTGGCACTCTTACCGTTACTGTTTACCCTGT  
22 N K V K Q S T A I A L A L L P L L F T P V

2581 GACAAAGCCCGGACACAGGAATGCCCTGTTCTGGAAAACCCGGCTGCTCAGGCGGATAT  
42 T K A R T P E M P V L E N R A Q G D I

2641 TACTGCACCCGGCGGTGCTCGCGTTTAAACGGGTGATCAGACTGCCGCTCTGCGTGATTC  
62 T A P G G A R R L T G D Q T A A L R D S

2701 TCCTTAGCGATAAACCTGCAAAAATATTATTTTGCTGATTGGCGATGGGATGGGGGACTC  
82 L S D K P A K N I I L L I G D G M G D S

2761 GGAAATTACTGSCCGCAGTAATTATGCCGAGGTGCGGGCGGCTTTTTAAAGGTATAGA  
102 E I T A C A A R N Y A E G A G G G F F K G I

2821 TGCCTTACCGCTTACCGGGCAATCACTCACTATGCGCTGAATAAAAAAACCGGCAAAACC  
122 A L P L T G Q Y T H Y A L N K K T G K P

2881 GGACTACGTCACCGACTCGGCTGCATCAGCAACCGCGCTGCTCAACCGGTGTCAAAACCTA  
142 D Y V T D S A S A S A T A G S T G V K T Y

2941 TAACGGCGCGCTGGCGCTCGATATTCCGAAAAAGATCACCACGATTCTGAAATGGC  
162 N G A L G V D I H E K D H P T I L E M A

3001 AAAAGCCGAGGTCTGGCGACCGGTAACGTTTCTACCGCAGAGTTGCAGGATGCCACGCC  
182 K A A G L A T G N V S T A E L Q D A T P

3061 CGCTCGCGTGGTGGCAGATGTGACCTGCGGAAATGCTACCGTCCGAGCGGACAGTGA  
202 A L V A H V T S R K C Y G P S A T S E

3121 AAAATGTCGGGTAAACGCTCTGGAAAAAGCGGAAAAGGATCGATTACGAAACCGCTGCT  
222 K C P G N A L E K G G K G S I T E Q L L

3181 TAACGCTCGTGGCGACGTTTACGCTTGGCGGCGGCGAAAAACCTTTGCTGAACCGGCAAC  
242 N A R A D V T L G G G A K T F A E T A T

3241 CGCTGGTGAATGGCAGGAAAAACGCTGCGTGAACAGGACAGGCGCTGGTTTACGTT  
262 A G E W Q G K T L R E Q Q A Q A R G Y Q L

3301 GGTGAGCGATGCTGCCTCACTGAATTCGGTGAACGAAGCGAATCAGCAAAACCCCTGCCT  
282 V S D A A S L N S V T E A N Q Q K P L L

3361 TGGCCTGTTTGTCTGACGGCAATATGCCAGTGCCTGGCTAGGACCGGAAAGCAACGTACCA  
302 G L F A D G N M P V R W L G P K A T Y H

3421 TGGCAATATCGATAAGCCCGCAGTCACTCTACGCCAAATCCGCAACGTAATGACAGTGT  
322 G N I D K P A V T C T C P N Q R N D S V

3481 ACCAACCTTGGCGCAGATGACGCAAAAGCCATTGAATTGTTGAGTAAAAATGAGAAAG

WO 03/072014

PCT/US02/16877

342 P T L A Q M T D K A I E L L S K N E K G  
3541 CTTTTCCTGCAAGTTGAAGGTGCGTCAATCGATAAACAGGATCATGCTGCGAATCCCTTG  
5 362 F F L Q V E G A S I D K Q D H A A N P C  
3601 TGGGCAAAATTGGCGAGACGCTCGATCTCGATGAAGCCGTACAACGGGCGCTGGAATTCGC  
382 G Q I G E T V D L D E A V Q R A L E F A  
3661 TAAAAAGGAGGGTAAACACGCTGGTCATAGTCACCGCTGATCACGCCACGCCAGCCAGAT  
10 402 K K E G N T L V I V T A D H A H A S Q I  
3721 TGTTGCGCCGGATACCAAAGCTCCGGCCCTCACCAGGCGCTAAATACCAAGATGGCGC  
422 V A P D T K A P G L T Q A L N T K D G A  
3781 AGTGATGGTGATGAGTTACGGGAATCCGAAGAGGATTACAAGAACATACCGGCAGTCA  
15 442 V M V M S Y G N S E E D S Q E H T G S Q  
3841 GTTGCCTATTGCGCGTATGGCCCGCATGCCGCCAATGTTGTTGGACTGACCGACCAAGAC  
20 462 L R I A A Y G P H A A N V V G L T D Q T  
3901 CGATCTCTTCTACACCATGAAAGCCGCTCTGGGGCTGAAATCTCTAGA XbaI  
482 D L F Y T M K A A L G L K S R

25 Complete *PhoA* from *E. coli* MG1655 cloned into pMPX-5. Create chimeric fusions with the *phoA* by cloning into XbaI and introducing a stop sequence.

30 SEQ ID NO 24

pMPX-62 (MalE residues 1-28 cloned into pMPX-5 using PCR-introduced PstI and XbaI)

35 Shine-Delgarno PstI  
2401 GAATTGAGCGCTTTTTAGACTGGTCGTAATGAAATTCAGCAGGATCACATTCTGCAGAT M  
1  
2461 GAAAAATAAAACAGGTGCACGCATCCTCGCATTATCCGCATTAAACGACGATGATGTTTTTC  
40 2 K I K T G A R I L A L S A L T T M M F S  
2521 CGCCTCGGCTCTCGCCAAATCTCTAGA XbaI  
22 A S A L A K I S R

45 MalE residues 1-28 from *E. coli* MG1655 cloned into pMPX-5. Create chimeric fusions with the *malE* by cloning into XbaI and introducing a stop sequence.

50 SEQ ID NO 25

pMPX-61 (MalE residues 1-370 cloned into pMPX-5 using PCR-introduced PstI and XbaI)

55 Shine-Delgarno PstI  
2401 GAATTGAGCGCTTTTTAGACTGGTCGTAATGAAATTCAGCAGGATCACATTCTGCAGAT

PCT/US02/16877

XbaI

WO 03/072014

PCT/US02/16877

MalE residues 1-370 from *E. coli* MG1655 cloned into pMPX-5. Create chimeric fusions with the *malE* by cloning into XbaI and introducing a stop sequence.

5

SEQ ID NO 26

10 pMPX-17 (complete *tig* and *groESL*, both with complete native control region cloned into pMPX-5 using PCR-introduced NarI and HindIII. The *tig* and *groESL* regions are joined using XbaI). Construct to be used on same vector as protein to be expressed or as a template for insertion into pACYC184.

15

NarI

181 ACCATATGCG GTGTGAAATA CCGCACAGAT GCGTAAGGAG AAAATACCGC  
ATCAGGCGCC

20

241 ATACGCGACA GCGCGCAATA ACCGTTCTCG ACTCATAAAA GTGATGCCGC  
TATAATGCCG  
301 CGTCTATTT GAATGCTTTC GGGATGATTC TGGTAAACAGG GAATGTGATT  
GATTATAAGA  
361 ACATCCCGGT TCCGCGAAGC CAACAACCTG TGCTTCGGGG GTAAGAGTTG  
ACCGAGCACT

25

421 GTGATTTTTT GAGGTAACAA <sup>+1 *tig*</sup> GATGCAAGTT TCAGTTGAAA CCACTCAAGG  
CCTTGCCCGC

→

30

481 CGTGTAAACGA TTAATATCGC TGCTGACAGC ATCGAGACCG CTGTTAAAGG  
CGAGCTGGTC  
541 AACGTTGCGA AAAAAGTAAG TATTGACGGC TTCCGCAAGG GCAAAAGTCC  
AATGAATATC  
35 601 GTTGCTCAGC GTTATGGCGC GTCTGTACGC CAGGACGTC TGGGTGACCT  
GATGAGCCGT  
661 AACTTCATTG ACGCCATCAT TAAAGAAAAA ATCAATCCGG CTGGCGCACC  
GACTTATGTT  
40 721 CCGGGCGAAT ACAAGCTGGG TGAAGACTTC ACITACTCTG TAGAGTTTGA  
AGTTTATCCG  
781 GAAGTTGAAC TGCAGGCTCT GGAAGCGATC GAAGTTGAAA AACCGATCGT  
TGAAGTGACC  
841 GACGCTGACG TTGACGGCAT GCTTGATACT CTGCGTAAAC AGCAGGCGAC  
CTGGAAGAA  
45 901 AAAGACGCGC CTGTTGAAGC AGAAGACCGC GTAACCATCG ACTTCACCGG  
TTCGTAGAC  
961 GGCGAAGAGT TCGAAGCGCG TAAAGCGTCT GATTTCGTAC TGGCGATGGG  
CCAGGTCGT  
1021 ATGATCCCGG GCTTTGAAGA CGGTATCAAA GGCCCAAAAG CTGGCGAAGA  
GTTCAACCATC  
50 1081 GACGTGACCT TCCCGGAAGA ATACCACGCA GAAACCTGTA AAGGTAAGAC  
AGCGAAATTC  
1141 GCTATCAACC TGAAGAAAGT TGAAGAGCGT GAACTGCCGG AACTGACTGC  
AGAATTCATC  
55 1201 AAACGTTTTC GCGTTGAAGA TGTTTCCGTA GAAGGTCGTC GCGCTGAAGT  
GCGTAAAAAC  
1261 ATGGAGCGCG AGCTGAAGAG CGCCATCCGT AACCGCGTTA AGTCTCAGGC  
GATCGAAGGT

WO 03/072014

PCT/US02/16877

1321 CTGGTAAAAG CTAACGACAT CGACGTACCG GCTGCGCTGA TCGACAGCGA  
AATCGACGTT  
1381 CTGCGTCGCC AGGCTGCACA GCGTTTCGGT GGCAACGAAA AACAAAGCTCT  
GGAACGCGG  
5 1441 CCGCAACTGT TCGAAGAACA GGCTAAACGC CGCGTAGTTG TTGGCTGCT  
GCTGGGCGAA  
1501 GTTATCGCGA CCAACGAGCT GAAAGCTGAC GAAGAGCGCG TGAAGGCCT  
GATCGAAGAG  
1561 ATGGCTTCTG CGTACGAAGA TCCGAAAGAA GTTATCGAGT TCTACAGCAA  
10 AAACAAAGAA  
1621 CTGATGGACA ACATGCGCAA TGTGCTCTG GAAGAACAGG CTGTTGAAGC  
TGTACTGGCG  
15 *tig* Stop  
1681 AAAGCGAAAG TGACTGAAAA AGAAACCACT TTCAACGAGC TGATGAACCA  
GCAGGCCTAA  
20 1741 TAATAATCTA GAGGTAGCAC AATCAGATTG GCTTATGACG GCGATGAAGA  
AATTGCGATG  
1801 AAATGTCAGG TGAATCAGGG TTTTCACCCG ATTTGTGCT GATCAGAATT  
TTTTTCTCTT  
1861 TTCCCCCTTG AAGGGGCGAA GCCTCATCCC CATTTCTCTG GTCACCAGCC  
25 GGGAAACCAC  
  
+1  
*groES*  
1921 GTAAGCTCCG GCGTCACCCA TAACAGATAC GGACTTTCTC AAAGGAGAT  
30 TATCAATGAA  
→  
1981 TATTCGTCCA TTGCATGATC GCGTGATCGT CAAGCGTAAA GAAGTGAATA  
CTAAATCTGC  
35 2041 TGGCGGCATC GTTCTGACCG GCTCTGCAGC GGCTAAATCC ACCCGCGCG  
AAGTGCTGCG  
2101 TGTGGCAAT GGCCTATCC TTGAAAAATG CGAAGTGAAG CCGCTGGATG  
TGAAAGTTGG  
2161 CGACATGCTT ATTTTCAACG ATGGCTACGG TGTGAATCT GAGAAGATCG  
40 ACAATGAAGA  
Stop *groES*  
2221 AGTGTGATC ATGTCGAAA GCGACATTCT GGCAATTGTT GAAGCGTAAT  
CGCGCACGA  
45 +1 *groEL*  
2281 CACTGAACAT ACGAATTTAA GGAATAAGA TAATGCCAGC TAAAGACGTA  
AAATTCGGTA  
→  
50 2341 ACGACGCTCG TGTGAAAATG CTGCGCGCGG TAAACGTACT GGCAGATGCA  
GTGAAAGTTA  
2401 CCCTCGGTCC AAAAGGCCGT AACGTAGTTC TGGATAAATC TTTCGGTGCA  
CGACCATCA  
2461 CCAAAGATGG TGTTCCTGTT GCTCGTGAAA TCGAACTGGA AGACAAGTTC  
55 GAAATATGG  
2521 GTGCGCAGAT GGTGAAAGAA GTTGCTCTTA AAGCAAACGA CGCTGCAGGC  
GACGGTACCA  
2581 CCACTGCAAC CGTACTGGCT CAGGCTATCA TCACTGAAGG TCTGAAAGCT  
GTTGCTGCGG

WO 03/072014

PCT/US02/16877

```

2641 GCATGAACCC GATGGACCTG AAACGTGCTA TCGACAAAGC GGTACCGCT
GCAGTTGAAG
2701 AACTGAAAGC GCTGTCCGTA CCATGCTCTG ACTCTAAAGC GATTGCTCAG
GTTGGTACCA
5 2761 TCTCCGCTAA CTCCGACGAA ACCGTAGGTA AACTGATCGC TGAAGCGATG
GACAAAGTCG
2821 GTAAAGAAGG CGTTATCACC GTTAAAGACG GTACCGGTCT GCAGGACGAA
CTGGACGTGG
10 2881 TTGAAGGTAT GCAGTTCGAC CGTGGCTACC TGTCTCCTTA CTTTCATCAAC
AAGCCGGAAA
2941 CTGGCGCAGT AGAACTGGAA AGCCCGTTCA TCCTGCTGGC TGACAAGAAA
ATCTCCACCA
3001 TCCGCGAAAT GCTGCCGGTT CTGGAAGCTG TTGCCAAAGC AGGCAAAACG
CTGCTGATCA
15 3061 TCGCTGAAGA TGTAAGAAGC GAAGCGCTGG CAATCTCGGT TGTTAACACC
ATGCGTGGCA
3121 TCGTGAAAGT CGCTGCGGTT AAAGCACCGG GCTTGGCGCA TCGTCGTAAA
GCTATGCTGC
181 AGGATATCGC AACCCTGACT GCGCGTACCG TGATCTCTGA AGAGATCGGT
20 ATGGAGCTGG
3241 AAAAAGCAAC CCTGGAAGAC CTGGGTGAGG CTAAACGTGT TGTGATCAAC
AAAGACACCA
3301 CCACTATCAT CGATGGCGTG GGTGAAGAAG CTGCAATCCA GGGCCGTGTT
GCTCAGATCC
25 3361 GTCAGCAGAT TGAAGAAGCA ACTTCTGACT ACGACCGTGA AAAACTGCAG
GAACGCGTAG
3421 CGAAACTGGC AGGCGGCGTT GCAGTTATCA AAGTGGGTGC TGCTACCGAA
GTTGAAATGA
3481 AAGAGAAAAA AGCACGCGTT GAAGATGCCC TGCAACGCAC CCGTGTCTGC
30 GTAGAAGAAG
3541 GCGTGGTTGC TGGTGGTGGT GTTGCGCTGA TCCGCGTAGC GTCTAAACTG
GCTGACCTGC
3601 GTGGTCAGAA CGAAGACCAG AACGTGGGTA TCAAAGTTGC ACTGCGTGCA
ATGGAAGCTC
35 3661 CGCTGCGTCA GATCGTATTG AACTGCGGCG AAGAACCGTC TGTGTTGCT
AACACCGTTA
3721 AAGGCGGCGA CGGCAACTAC GGTACAAACG CAGCAACCGA AGAATACGGC
AACATGATCG
3781 ACATGGGTAT CCTGGATCCA ACCAAAGTAA CTCGTTCTGC TCTGCAGTAC
40 GCAGCTTCTG
3841 TGGCTGGCCT GATGATCACC ACCGAATGCA TGGTTACCGA CCGCCGAAA
AACGATGCAG

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Stop

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45 groEL
3901 CTGACTTAGG CGCTGCTGGC GGTATGGCG GCATGGGTGG CATGGCGGC
ATGATGTAAT
HindIII
50 3961 AATAAGCTTG CATGCCTGCA GGTGACTCT AGAGGATCCC CGGGTACCGA
GCTCGAATTC

```

55 SEQ ID NO 27

pMPX-63 (C-terminal fusion with Factor Xa TrxA residues 2-109 FLAG cloned into pMPX-5 using PCR-introduced PstI and BamHI)

WO 03/072014

PCT/US02/16877

2401 Shine-Delgarno PstI  
1 GAATTCAGGCGCTTTTITAGACTGGTCGTAATGAAATTCAGCAGGATCACATTCTGCAGATM

5 Factor Xa XbaI XhoI  
2461 GATCGAAGCCCGCTCTAGACTCGAGAGCGATAAAATTATTCACTGACTGACGACAGTTT  
2 I E A R S R L E S D K I I H L T D D S F

2521 TGACACGGATGTACTCAAAGCGGACGGGGCGATCCTCGTCGATTTCTGGGCAGAGTGGTG  
10 22 D T D V L K A D G A I L V D F W A E W C

2581 CGGTCCGTGCAAAATGATCGCCCGATTCTGGATGAAATCGCTGACGAATATCAGGGCAA  
42 G P C K M I A P I L D E I A D E Y Q G K

2641 ACTGACCGTTGCAAACTGAACATCGATCAAAACCTGGCACTGCGCCGAAATATGGCAT  
15 62 L T V A K L N I D Q N P G T A P K Y G I

2701 CCGTGGTATCCGACTCTGCTGTTTCAAAAACGGTGAAGTGGCGGCAACCAAGTGGG  
20 82 R G I P T L L L F K N G E V A A T K V G

2761 XhoI  
102 TGCACGTCTAAAGGTCAGTTGAAAGAGTTCCCTCGACGCTAACCTGGCGCTCGAGGATTA  
A L S K G Q L K E F L D A N L A L E D Y

25 BamHI  
2821 TAAAGATCATGATGGCGATTATAAAGATCATGATGATTAATAAGGATCCCGGGTACCGA  
122 K D H D G D Y K D H D D

30

Gene *trxA* (2-109) from *E. coli* MG1655 cloned into pMPX-5. Create chimeric fusions with the *trxA* by cloning into PstI and XbaI. May remove *trxA* using XhoI. FLAG sequence shown in italics only.

35

SEQ ID NO:28

Rat Edg-3 nucleotide sequence

ATGGCAACCACGCACGCGCAGGGGACCCGCCAGTCTTGGGGAAT  
GATACTCTCCGGGAACATTATGATTACGTGGGGAAGCTGGCAGGCAGGCTGCGGG  
ATCCCCCTGAGGGTAGCAACCCTATCACCACCATCCTCTCTTGCTACCTGTAGC  
40 TTCATCGTCTTGGAAGAACCTGATGGTITTTGATTGCCATCTGGAAAAACAATAAAT  
TCATAACCGCATGTACTTTTTCATCGGCAACTTGGCTCTCTGCGACCTGCTGGCCG  
GCATAGCCTACAAGGTCAACATTTCTGATGTCCGGTAGGAAGACGTTACGCCTGTC  
TCCAACAGTGTGGTTCTCTCAGGGAGGGCAGTATGTTCTGATGCCCTGGGCGCATCC  
ACATGACAGCTTATTGGCCATTGCCATTGAGCGGCACCTGACCATGATCAAGATGA  
45 GGCCGTACGACGCCAACAAGAAGCACCGCGTGTCTCTCTGATTGGGATGTGCTG

WO 03/072014

PCT/US02/16877

GCTAATTGCCTTCTCGCTGGGTGCCCTGCCCATCCTGGGCTGGAAGTGCCTGGAGA  
ACTTTCCCGACTGCTCTACCATCTTGCCCTCTACTCCAAGAAATACATTGCCTTT  
CTCATCAGCATCTTCACAGCCATTCTGGTGACCATCGTCATCTTGTACGCGCGCAT  
CTACTTCCTGGTCAAGTCCAGCAGCCGCAAGGTGGCCAACCACTCCGAGAGA  
5 TCCATGGCCCTTCTGCGGACCGTAGTGATCGTGGTGAGCGTGTTCAATCGCCTGTTG  
GTCCCCCTTTTCATCCTCTTCTCTATCGATGTGGCTGCAGGGCGAAGGAGTGCT  
CCATCCTCTTCAAGAGTCAAGTGGTTCATCATGCTGGCTGTCTCAACTCGGCCATG  
AACCCTGTCTATACAGCTGGCCAGCAAAGAGATGCGGCGTGCTTTCTTCGGGT  
GGTGTGCGGTGTCTGGTCAAGGGCAAGGGGACCCAGGCTCCCCGATGCAGCCT  
10 GCTCTTGACCCGAGCAGAAGTAAATCAAGCTCCAGTAAACAACAGCAGCAGCCACT  
CTCCAAAGGTCAAGGAAGACCTGCCCCATGTGGCTACCTCTTCTGCGTCACTGA  
CAAAACGAGGTCGCTTCAGAATGGGGTCCTCTGCAAGTGA -1145

SEQ ID NO:29

15 Rat Edg-3 amino acid sequence

M A T T H A Q G H P P V L G N D T L R E H Y D Y V G K L A G R L  
R D P P E G S T L I T T I L F L V T C S F I V L E N L M V L I A  
I W K N N K F H N R M Y F F I G N L A L C D L L A G I A Y K V N  
I L M S G R K T F S L S P T V W F L R E G S M F V A L G A S T C  
20 S L L A I A I B R H L T M I K M R P Y D A N K K H R V F L L I G  
M C W L I A F S L G A L P I L G W N C L E N F P D C S T I L P L  
Y S K K Y I A F L I S I F T A I L V T I V I L Y A R I Y F L V K  
S S S R R V A N H N S E R S M A L L R T V V I V S V F I A C W  
S P L F I L F L I D V A C R A K E C S I L F K S Q W F I M L A V  
25 L N S A M N P V I Y T L A S K E M R R A F F R L V C G C L V K G  
K G T Q A S P M Q P A L D P S R S K S S S S N N S S S H S P K V  
K E D L P H V A T S S C V T D K T R S L Q N G V L C K

WO 03/072014

PCT/US02/16877

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SEQ ID NO.: 153

5 pMPX-66 arabinose-inducible expression vector

1 TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT  
GCAGCTCCCG GAGACGGTCA

61 CAGCTTGTTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG  
10 TCAGGGCGCG TCAGCGGGTG

121 TTGGCGGGTG TCGGGGCTGG CTAACTATG CGGCATCAGA  
GCAGATTGTA CTGAGAGTGC

181 ACCATATGCG GTGTGAAATA CCGCACAGAT GCGTAAGGAG  
AAAATACCGC ATCAGGCGCC

15 241 ATTCGCCATT CAGGCTGCGC AACTGTTGGG AAGGGCGATC  
GGTGCGGGCC TCTTCGTAT

301 TACGCCAGCT GGCGAAAAGG GGATGTGCTG CAAGGCGATT  
AAGTTGGTA ACGCCAGGGT

20

HindIII

361 TTTCACAGTC ACGACGTTGT AAAACGACGG CCAGTGCCAA  
GCTTCAAGCC GTCAATTGTC

Stop araC

WO 03/072014

PCT/US02/16877

421 TGATTCGTTA CCAATTATGA CAACTTGACG GCTACATCAT  
TCACITTTTTC TTCACAACCG

5 481 GCACGGAAC TCGCTCGGGCT GGCCCCGGTG CATTTTTTAA  
ATACCCGCGA GAAATAGAGT

541 TGATCGTCAA AACCAACATT GCGACCGACG GTGGCGATAG  
GCATCCGGGT GGTGCTCAA

601 AGCAGCTTCG CCTGGCTGAT ACGTTGGTCC TCGCGCCAGC  
10 TTAAGACGCT AATCCCTAAC

661 TGCTGGCGGA AAAGATGTGA CAGACGCGAC GGCGACAAGC  
AAACATGCTG TCGACGCTG

721 GCGATATCAA AATTGCTGTC TGCCAGGTGA TCGCTGATGT  
ACTGACAAGC CTCGCGTACC

15 781 CGATTATCCA TCGGTGGATG GAGCGACTCG TTAATCGCTT  
CCATGCGCCG CAGTAACAAT

841 TGCTCAAGCA GATTTATCGC CAGCAGCTCC GAATAGCGCC  
CTTCCCCCTTG CCCGGCGTTA

901 ATGATTTGCC CAAACAGGTC GCTGAAATGC GGCTGGTGCG  
20 CTTCATCCGG GCGAAAGAAC

961 CCCGTATTGG CAAATATTGA CGGCCAGTTA AGCCATTTCAT  
GCCAGTAGGC GCGCGGACGA

1021 AAGTAAACCC ACTGGTGATA CCATTGCGGA GCCTCCGGAT  
GACGACCGTA GTGATGAATC

25 1081 TCTCCTGGCG GGAACAGCAA AATATCACCC GGTCGGCAAA  
CAAATTCTCG TCCCTGATTT

WO 03/072014

PCT/US02/16877

1141 TTCACCACCC CCTGACCGCG AATGGTGAGA TTGAGAATAT  
AACCTTTCAT TCCCAGCGGT

1201 CGGTCGATAA AAAAATCGAG ATAACCGTTG GCCTCAATCG  
GCGTTAAACC CGCCACCAGA

5 1261 TGGGCATTAA ACGAGTATCC CGGCAGCAGG GGATCATTTT  
GCGCTTCAGC CATACTTTTC

Start araC

1321 ATACTCCCGC CATTGAGAGA AGAAACCAAT TGTCCATATT  
10 GCATCAGACA TTGCCGTCAC

&lt;--

1381 TGCCTCTTTT ACTGGCTCTT CTCGCTAACC AAACCGGTAA  
CCCCGCTTAT TAAAAGCATT

15 1441 CTGTAACAAA GCGGGACCAA AGCCATGACA AAAACGCGTA  
ACAAAAGTGT CTATAATCAC

1501 GGCAGAAAAG TCCACATTGA TTATTTGCAC GGCCTCACAC  
TTTGCTATGC CATAGCATTT

1561 TTATCCATAA GATTAGCGGA TCCTACCTGA CGCTTTTTAT  
20 CGCAACTCTC TACTGTTTCT

SD SalI XbaI

1621 CCATACCCGT TTTTTTGGGC TAGCAGGAGG CCGTCGACTC  
TAGAGGATCC CCGCGCCCTC

25

WO 03/072014

PCT/US02/16877

Stem-loop

KpnI

1681 ATCCGAAAGG GCGTATTGGT ACCGAGCTCG AATTCGTAAT  
CATGGTCATA GCTGTTTCCT

5

1741 GTGTGAAATT GTTATCCGCT CACAATTCCA CACAACATAC  
GAGCCGGAAG CATAAAGTGT

1801 AAAGCCTGGG GTGCCTAATG AGTGAGCTAA CTCACATTAA  
TTGCGTTGCG CTCACTGCCC

10 1861 GCTTTCCAGT CGGGAAACCT GTCGTGCCAG CTGCATTAAT  
GAATCGGCCA ACGCGCGGGG

1921 AGAGGCGGTT TCGTATTGG GCGCTCTCC GCTTCTCGC  
TCACTGACTC GCTGCGCTCG

15 1981 GTCGTTCGGC TCGGGCGAGC GGTATCAGCT CACTCAAAGG  
CGGTAATACG GTATCCACA

2041 GAATCAGGGG ATAACGCAGG AAAGAACATG TGAGCAAAAG  
GCCAGCAAAA GGCCAGGAAC

2101 CGTAAAAAGG CCGCGTTGCT GCGGTTTTTC CATAGGCTCC  
GCCCCCTGA CGAGCATCAC

20 2161 AAAAATCGAC GCTCAAGTCA GAGGTGGCGA AACCCGACAG  
GACTATAAAG ATACCAGGCG

2221 TTTCCCCCTG GAAGCTCCCT CGTGCCTCTT CCTGTTCCGA  
CCCTGCCGCT TACCGGATAC

25 2281 CTGTCCGCCT TTCTCCCTTC GGGAAGCGTG GCGCTTTCTC  
ATAGTCACG CTGTAGGTAT

WO 03/072014

PCT/US02/16877

- 2341 CTCAGITCGG TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG  
TGCACGAACC CCCC GTTCAG
- 2401 CCCGACCGCT GCGCCTTATC CGGTA ACTAT CGTCTTGAGT  
CCAACCCGGT AAGACACGAC
- 5 2461 TTATCGCCAC TGGCAGCAGC CACTGGTAAC AGGATTAGCA  
GAGCGAGGTA TGTAGGCGGT
- 2521 GCTACAGAGT TCTTGAAGTG GTGGCCTAAC TACGGCTACA  
CTAGAAGGAC AGTATTGGT
- 2581 ATCTGCGCTC TGCTGAAGCC AGTTACCTTC GGAAAAAGAG  
10 TTGGTAGCTC TTGATCCGGC
- 2641 AAACAAACCA CCGCTGGTAG CGGTGGTTTT TTTGTTTGCA  
AGCAGCAGAT TACGCGCAGA
- 2701 AAAAAAGGAT CTCAAGAAGA TCCTTTGATC TTTCTACGG  
GGTCTGACGC TCAGTGGAAC
- 15 2761 GAAAACTCAC GTTAAGGGAT TTTGGTCATG AGATTATCAA  
AAAGGATCTT CACCTAGATC
- 2821 CTTTAAATT AAAAATGAAG TTTTAAATCA ATCTAAAGTA  
TATATGAGTA AACTTGGTCT
- 2881 GACAGTTACC AATGCITAAT CAGTGAGGCA CCTATCTCAG  
20 CGATCTGTCT ATTTCTGTTCA
- 2941 TCCATAGTTG CCTGACTCCC CGTCGTGTAG ATA ACTACGA  
TACGGGAGGG CTTACCATCT
- 3001 GGCCCCAGTG CTGCAATGAT ACCGCGAGAC CCACGCTCAC  
CGGCTCCAGA TTTATCAGCA
- 25 3061 ATAAACCAGC CAGCCGGAAG GGCCGAGCGC AGAAGTGGTC  
CTGCAACTTT ATCCGCCTCC

WO 03/072014

PCT/US02/16877

- 3121 ATCCAGTCTA TTAATTGTTG CCGGGAAGCT AGAGTAAGTA  
GTTCCGCAGT TAATAGTTTG
- 3181 CGCAACGTTG TTGCCATTGC TACAGGCATC GTGGTGTAC  
GCTCGTCGTT TGGTATGGCT
- 5 3241 TCATTACAGT CCGGTTCCCA ACGATCAAGG CGAGTTACAT  
GATCCCCAT GTTGTGCAAA
- 3301 AAAGCGGTTA GCTCCTTCGG TCCTCCGATC GTTGTGAGAA  
GTAAGTGGC CGCAGTGTTA
- 3361 TCACTCATGG TTATGGCAGC ACTGCATAAT TCTCTTACTG  
10 TCATGCCATC CGTAAGATGC
- 3421 TTTTCTGTGA CTGGTGAGTA CTCAACCAAG TCATTCTGAG  
AATAGTGAT GCGGCGACCG
- 3481 AGTTGCTCTT GCCCGGCGTC AATACGGGAT AATACCGCGC  
CATATAGCAG AACTTTAAAA
- 15 3541 GTGCTCATCA TTGAAAAACG TTCTTCGGGG CGAAAACTCT  
CAAGGATCTT ACCGCTGTTG
- 3601 AGATCCAGTT CGATGTAACC CACTCGTGCA CCCAACTGAT  
CTTCAGCATC TTTTACTTTC
- 3661 ACCAGCGTTT CTGGGTGAGC AAAAACAGGA AGGCAAAATG  
20 CCGCAAAAAA GGGAATAAGG
- 3721 GCGACACGGA AATGTGAAT ACTCATACTC TTCCTTTTTC  
AATATTATTG AAGCATTTAT
- 3781 CAGGGTATT GTTCATGAG CGGATACATA TTTGAATGTA  
TTTAGAAAAA TAAACAAATA
- 25 3841 GGGGTTCCGC GCACATTTC CCGAAAAGTG CCACCTGACG  
TCTAAGAAAC CATTATTATC

WO 03/072014

PCT/US02/16877

3901 ATGACATTAA CCTATAAAAA TAGGCGTATC ACGAGGCCCT  
TTCGTC

- 5 The segment araC through Para was taken from pBAD24 using PCR added HindIII and modified aligned Shine-Delgarno (SD) sequence with SalI followed by XbaI, a stem-loop transcriptional stop sequence, and KpnI. The PCR product was cloned into pUC18 using HindIII and KpnI.

10 SEQ ID NO.: 152

pMPX-72 rhamnose-inducible expression vector

- 1 TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT  
15 GCAGCTCCCG GAGACGGTCA
- 61 CAGCTTGCT GTAAAGCGGAT GCCGGGAGCA GACAAGCCCG  
TCAGGCGCG TCAGCGGGTG
- 121 TTGGCGGGTG TCGGGGCTGG CTTAACTATG CGGCATCAGA  
GCAGATTGTA CTGAGAGTGC
- 20 181 ACCATATGCG GTGTGAAATA CCGCACAGAT GCGTAAGGAG  
AAAATACCGC ATCAGGCGCC
- 241 ATTGCCATT CAGGCTGCGC AACTGTTGGG AAGGGCGATC  
GGTGCGGGCC TCTTCGCTAT
- 301 TACGCCAGCT GCGAAAGGG GGATGTGCTG CAAGGCGATT  
25 AAGTTGGGTA ACGCCAGGGT

WO 03/072014

PCT/US02/16877

Stop rhaR

361 TTTCCAGTC ACGACGTTGT AAAACGACGG CCAGTGCCAA  
CGTTAATTAA TCTTTCTGCG

5

HindIII

421 AATTGAGATG ACGCCACTGG CTGGGCGTCA TCCCGTTTC  
CCGGGTAAAC ACCACCGAAA

481 AATAGTTACT ATCTTCAAAG CCACATTCGG TCGAAATATC  
10 ACTGATTAAC AGGCGGCTAT

541 GCTGGAGAAG ATATTGCGCA TGACACACTC TGACCTGTGC  
CAGATATTGA TTGATGGTCA

601 TTCCAGTCTG CTGGCGAAAT TGCTGACGCA AAACGCGCTC  
ACTGCACGAT GCCTCATCAC

661 AAAATTTATC CAGCGCAAAG GGACTTTTCA GGCTAGCCGC  
15 CAGCCGGGTA ATCAGCTTAT

721 CCAGCAACGT TTCGCTGGAT GTTGGCGGCA ACGAATCACT  
GGTGTAACGA TGGCGATTCA

781 GCAACATCAC CAACTGCCCG AACAGCAACT CAGCCATTTT  
20 GTTAGCAAAC GGCACATGCT

841 GACTACTTTC ATGCTCAAGC TGACCGATAA CCTGCCGCGC  
CTGCGCCATC CCCATGTAC

901 CTAAGCGCCA GTGTGGTTGC CCTGCGCTGG CGTTAAATCC  
CGGAATCGCC CCCTGCCAGT

961 CAAGATTGAG CTTCAGACGC TCCGGGCAAT AAATAATATT  
25 CTGCAAAACC AGATCGTTAA

WO 03/072014

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1021 CGGAAGCGTA GGAGTGT TTA TCGTCAGCAT GAATGTAAAA  
GAGATCGCCA CGGGTAATGC

1081 GATAAGGGCG ATCGTTGAGT ACATGCAGGC CATTACCGCG  
CCAGACAATC ACCAGCTCAC

5 1141 AAAAATCATG TGTATGTTCA GCAAAGACAT CTTGCGGATA  
ACGGTCAGCC ACAGCGACTG

1201 CCTGCTGGTC GCTGGCAAAA AAATCATCTT TGAGAAGTTT  
TAACTGATGC GCCACCGTGG

1261 CTACCTCGGC CAGAGAACGA AGTTGATTAT TCGCAATATG  
10 CGGTACAAAT ACGTTGAGAA

Stop rhaS Start rhaR

1321 GATTGCGTT ATTGCAGAAA GCCATCCCGT CCCTGGCGAA  
TATCACGCGG TGACCAGTTA

15 <—

1381 AACTCTCGGC GAAAAAGCGT CGAAAAGTGG TTA CTGTGCGC  
TGAATCCACA GCGATAGGCG

1441 ATGTCAGTAA CGCTGGCCTC GCTGTGGCGT AGCAGATGTC  
20 GGGCTTTCAT CAGTCGCAGG

1501 CGGTTCAAGT ATCGCTGAGG CGTCAGTCCC GTTTGCTGCT  
TAAGTGCCG ATGTAGCGTA

1561 CGCAGTGAAA GAGAAAATTG ATCCGCCACG GCATCCCAAT  
TCACCTCATC GGCAAAATGG

25 1621 TCCTCCAGCC AGGCCAGAAG CAAGTTGAGA CGTGATGCGC  
TGTTTCCAG GTTCTCCTGC

WO 03/072014

PCT/US02/16877

1681 AAACGTCTTT TACGCAGCAA GAGCAGTAAT TGCATAAACA  
AGATCTCGCG ACTGGCGGTC

1741 GAGGGTAAAT CATTTTCCCC TTCCTGCTGT TOCATCTGTG  
CAACCAGCTG TCGCACCTGC

5 1801 TGCAATACGC TGTGGTTAAC GCGCCAGTGA GACGGATACT  
GCCCCCCAG CTCCTGTGGC

1861 AGCAACTGAT TCAGCCCGGC GAGAACTGA AATCGATCCG  
GCGAGCGATA CAGCACATTG

1921 GTCAGACACA GATTATCGGT ATGTTCATAC AGATGCCGAT  
10 CATGATCGCG TACGAAACAG

2081 ACCGTGCCAC CGGTGATGGT ATAGGGCTGC CCATTAAACA  
CATGAATACC CGTGCCATGT

2241 TCGACAATCA CAATTTCATG AAAATCATGA TGATGTTTCA  
GAAAAATCCG CTGCGGGAGC

15 2101 CGGGGTTCTA TCGCCACGGA CGCGTTACCA GACGGAAAAA  
AATCCCACT ATGTAATACG

Start rhaS

2161 GTCATACTGG CCTCCTGATG TCGTCAACAC GCGGAAATAG  
20 TAATCACGAG GTCAGGTTCT

&lt;--

2221 TACCTTAAAT TTTCGACGGA AAACCACGTA AAAAACGTCG  
ATTTTCAAG ATACAGCGTG

25 2281 AATTTTCAGG AAATGCGGTG AGCATCATAT CACCACAATT  
CAGCAAATTG TGAACATCAT

WO 03/072014

PCT/US02/16877

2341 CACGTTTCATC TTTCCCTGGT TGCCAATGGC CCATTTTCCT  
GTCAGTAACG AGAAGGTCGC

SD PstI SalI

5 2401 GAATTCAGGC GCTTTTTAGA CTGGTCGTAA TGAAATTCAG  
GAGGTTCTGC AGGTCGACTC

XbaI

Stem-loop

KpnI

2461 TAGAGGATCC CCGCGCCCTC ATCCGAAAGG GCGTATTGGT  
10 ACCGAGCTCG AATTCGTAAT

2521 CATGGTCATA GCTGTTTCCT GTGTGAAATT GTTATCCGCT  
CACAAATCCA CACAACATAC

2581 GAGCCGGAAG CATAAAGTGT AAAGCCTGGG GTGCCTAATG  
15 AGTGAGCTAA CTCACATTAA

2641 TTGCGTTGCG CTCACTGCCC GCTTTCCAGT CGGGAAACCT  
TGCTGTCCAG CTGCATTAAT

2701 GAATCGGCCA ACGCGCGGGG AGAGGCGGTT TGC GTATTGG  
GCGCTCTTCC GCTTCCTCGC

2761 TCACTGACTC GCTGCGCTCG GTCGTTCGGC TGCGGCGAGC  
20 GGATCAGCT CACTCAAAGG

2821 CGGTAATACG GTTATCCACA GAATCAGGGG ATAACGCAGG  
AAAGAACATG TGAGCAAAAG

2881 GCCAGCAAAA GGCCAGGAAC CGTAAAAAGG CCGCGTTGCT  
25 GGCGTTTTTC CATAGGCTCC

WO 03/072014

PCT/US02/16877

2941 GCCCCCTGA CGAGCATCAC AAAAATCGAC GCTCAAGTCA  
GAGGTGGCGA AACCCGACAG

3001 GACTATAAAG ATACCAGGCG TTTCCTCCCTG GAAGCTCCCT  
CGTGCGCTCT CCTGTTCOGA

5 3061 CCCTGCCGCT TACCGGATAC CTGTCCGCTT TTCTCCCTTC  
GGGAAGCGTG GCGCTTTCTC

3121 ATAGCTCAG CTGTAGGTAT CTCAGTTCGG TGTAAGTCGT  
TCGCTCCAAG CTGGGCTGTG

3181 TGCACGAACC CCCCCTTCAG CCCGACCGCT GCGCCTTATC  
10 CGTAACTAT CGTCTTGAGT

3241 CCAACCCGGT AAGACACGAC TTATCGCCAC TGGCAGCAGC  
CACTGGTAAC AGGATTAGCA

3301 GAGCGAGGTA TGTAAGCGGT GCTACAGAGT TCTTGAAGTG  
GTGGCCTAAC TACGGCTACA

15 3361 CTAGAAGGAC AGTATTTGGT ATCTGCGCTC TGCTGAAGCC  
AGTTACCTTC GGAAAAAGAG

3421 TTGGTAGCTC TTGATCCGGC AAACAAACCA CCGCTGGTAG  
CGGTGGTTTT TTTGTTTGCA

3481 AGCAGCAGAT TACGCGCAGA AAAAAAGGAT CTCAAGAAGA  
20 TCCTTTGATC TTTTCTACGG

3541 GGTCTGACGC TCAGTGGAAC GAAAACTCAC GTTAAGGGAT  
TTTGGTCATG AGATTATCAA

3601 AAAGGATCTT CACCTAGATC CTITTTAAAT AAAAAATGAAG  
TTTTAAATCA ATCTAAAGTA

25 3661 TATATGAGTA AACTTGGTCT GACAGTTACC AATGCTTAAT  
CAGTGAGGCA CCTATCTCAG

WO 03/072014

PCT/US02/16877

3721 CGATCTGTCT ATTTCGTTCA TCCATAGTTG CCTGACTCCC  
CGTCGTGTAG ATAACACGA

3781 TACGGGAGGG CTTACCATCT GGGCCAGTG CTGCAATGAT  
ACCGGAGAC CCACGCTCAC

5 3841 CGGCTCCAGA TTTATCAGCA ATAAACCAGC CAGCCGAAG  
GGCCGAGCG AGAAGTGGTC

3901 CTGCAACTTT ATCCGCCTCC ATCCAGTCTA TTAATTGTTG  
CCGGGAAGCT AGAGTAAGTA

3961 GTTCGCCAGT TAATAGTTTG CGCAACGTTG TTGCCATTGC  
10 TACAGGCATC GTGGTGTCAC

4021 GCTCGTCGTT TGGTATGGCT TCATTCAGCT CCGGTTCCCA  
ACGATCAAGG CGAGTTACAT

4081 GATCCCCAT GTTGTGCAAA AAAGCGGTTA GCTCCTTCGG  
TCTCCGATC GTTGTCAGAA

15 4141 GTAAGTTGGC CGCAGTGITA TCACTCATGG TTATGGCAGC  
ACTGCATAAT TCTCTTACTG

4201 TCATGCCATC CGTAAGATGC TTTTCTGTGA CTGGTGAGTA  
CTCAACCAAG TCATTCTGAG

4261 AATAGTGTAT GCGGCGACCG AGTTGCTCTT GCCCGGCGTC  
20 AATACGGGAT AATACGCGC

4321 CACATAGCAG AACTTTAAAA GTGCTCATCA TTGAAAAACG  
TTCTTCGGGG CGAAAACCT

4381 CAAGGATCTT ACCGCTGTTG AGATCCAGTT CGATGTAACC  
CACTCGTGCA CCAACTGAT

4441 CTTCAGCATC TTITACTTTC ACCAGCGTTT CTGGGTGAGC  
25 AAAAAACAGGA AGGCAAAATG

WO 03/072014

PCT/US02/16877

4501 CCGCAAAAAA GGGAATAAGG GCGACACGGA AATGTTGAAT  
ACTCATACTC TTCCTTTTTC

4561 AATATTATTG AAGCATTAT CAGGGTTATT GTCTCATGAG  
CGGATACATA TTTGAATGTA

5 4621 TTTAGAAAAA TAAACAAATA GGGGTTCGC GCACATTTCC  
CCGAAAAGTG CCACCTGACG

4681 TCTAAGAAAC CATTATTATC ATGACATTAA CCTATAAAAA  
TAGGCGTATC ACGAGGCCCT

4741 TTCGTC

10 The segment rhaR through Prha was taken from the E. coli chromosome using PCR  
added HindIII and modified aligned Shine-Delgarno (SD) sequence with PstI followed by  
SalI, XbaI, a stem-loop transcriptional stop sequence, and KpnI. The PCR product was  
cloned into pUC18 using HindIII and KpnI.

15

SEQ ID NO.: 151

20 pMPX-67 rhamnose-inducible expression vector

1 TC GCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT  
GCAGCTCCCC GAGACGGTCA

61 CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCC  
25 TCAGGGCGCG TCAGCGGGTG

WO 03/072014

PCT/US02/16877

121 TTGGCGGGTG TCGGGGCTGG CTTAACTATG CGGCATCAGA  
GCAGATTGTA CTGAGAGTGC

181 ACCATATGCG GTGTGAAATA CCGCACAGAT GCGTAAGGAG  
AAAATACCGC ATCAGGCGCC

5 241 ATTCGCCATT CAGGCTGCGC AACTGTTGGG AAGGGCGATC  
GGTGCGGGCC TCTTCGTAT

301 TACGCCAGCT GGCGAAAGGG GGATGTGCTG CAAGGCGATT  
AAGTTGGGTA ACGCCAGGGT

10 Stop rhaR

361 TTTCCAGTC ACGACGTTGT AAAACGACGG CAGTGCCAA  
GCTTAATTAA TCTTTCTGCG

HindIII

15 421 AATTGAGATG ACGCCACTGG CTGGGCGTCA TCCCGGTTTC  
CCGGGTAAAC ACCACGAAA

481 AATAGTTACT ATCTCAAAG CCACATTCGG TCGAAATATC  
ACTGATTAAC AGGCGGCTAT

541 GCTGGAGAAG ATATTGCGCA TGACACACTC TGACCTGTGC  
20 CAGATATTGA TTGATGGTCA

601 TTCCAGTCTG CTGGCGAAAT TGCTGACGCA AAACGCGCTC  
ACTGCACGAT GCCTCATCAC

661 AAAATTTATC CAGCGCAAAG GGACTTTTCA GGCTAGCCGC  
CAGCCGGGTA ATCAGCTTAT

25 721 CCAGCAACGT TTCGCTGGAT GTTGCGGCA ACGAATCACT  
GGTGTAACGA TGGCGATTCA

WO 03/072014

PCT/US02/16877

781 GCAACATCAC CAACTGCCCG AACAGCAACT CAGCCATTTC  
GTTAGCAAAC GGCACATGCT

841 GACTACTTTC ATGCTCAAGC TGACCGATAA CCTGCCGCGC  
CTGCGCCATC CCCATGCTAC

5 901 CTAAGCGCCA GTGTGGTTGC CCTGCGCTGG CGTTAAATCC  
CGGAATCGCC CCTTGCCAGT

961 CAAGATTGAG CTTGAGACGC TCCGGGCAAT AAATAATATT  
CTGCAAAACC AGATCGTTAA

1021 CGGAAGCGTA GGAGTGTTTA TCGTCAGCAT GAATGTAAAA  
10 GAGATCGCCA CGGGTAATGC

1081 GATAAGGGCG ATCGTTGAGT ACATGCAGGC CATTACCGCG  
CCAGACAATC ACCAGCTCAC

1141 AAAAATCATG TGTATGTTCA GCAAAGACAT CTTGCGGATA  
ACGGTCAGCC ACAGCGACTG

15 1201 CTTGCTGGTC GCTGGCAAAA AAATCATCTT TGAGAAGTTT  
TAACTGATGC GCCACCGTGG

1261 CTACCTCGGC CAGAGAACGA AGTTGATTAT TCGCAATATG  
GCGTACAAAT ACGTTGAGAA

20 Stop rhaS Start rhaR

1321 GATTGCGTT ATTGCAGAAA GCCATCCCGT CCCTGGCGAA  
TATCACGCGG TGACCAGTTA

<--

25 1381 AACTCTCGGC GAAAAAGCGT CGAAAAGTGG TTACTGTCGC  
TGAATCCACA GCGATAGGCG

PCT/US02/16877

25

63/268

WO 03/072014

PCT/US02/16877

2161 GTCATACTGG CCTCCTGATG TCGTCAACAC GGCGAAATAG  
TAATCACGAG GTCAGGTTCT

&lt;--

5 2221 TACCTTAAAT TTTGACGGA AAACCACGTA AAAAACGTCG  
ATTTTCAAG ATACAGCGTG

2281 AATTTTCAGG AAATGCGGTG AGCATCACAT CACCACAATT  
GAGCAAATTG TGAACATCAT

2341 CACGTTTCATC TTTCCCTGGT TGCCAATGGC CCATTTTCCT  
10 GTCAGTAACG AGAAGGTCGC

SD Sall XbaI

2401 GAATTCAGGC GCTTTTLAGA CTGGTCGTAA TGAAATTCAG  
15 GAGGTTGTCTG ACTCTAGAGG

Stem-loop

KpnI

2461 ATCCCCGCGC CCTCATCCGA AAGGGCGTAT TGGTACCGAG  
CTCGAATTCTG TAATCATGGT

20

2521 CATAGCTGTT TCCTGTGTGA AATTGTTATC CGCTCACAAT  
TCCACACAAC ATACGAGCCG

2581 GAAGCATAAA GTGTAAGGCC TGGGGTGCCT AATGAGTGAG  
25 CTAATCACA TTAATTGCGT

WO 03/072014

PCT/US02/16877

2641 TGCCTCACT GCCCGCTTTC CAGTCGGGAA ACCTGTCGTG  
CCAGCTGCAT TAATGAATCG

2701 GCCAACGCGC GGGGAGAGGC GGTTCGCTA TTGGGCGCTC  
TTCCGCTTCC TCGCTCACTG

5 2761 ACTCGCTGCG CTCGGTCGTT CGGCTGCGGC GAGCGGTATC  
AGCTCACTCA AAGGCGGTAA

2821 TACGGTTATC CACAGAATCA GGGGATAACG CAGGAAAGAA  
CATGTGAGCA AAAGGCCAGC

10 2881 AAAAGGCCAG GAACCGTAAA AAGCCCGCT TGCTGGCGTT  
TITCCATAGG CTCGCCCCC

2941 CTGACGAGCA TCACAAAAAT CGACGCTCAA GTCAGAGGTG  
GCGAAACCCG ACAGGACTAT

3001 AAAGATACCA GCGTTTTCCC CCTGGAAGCT CCCTCGTGCG  
CTCTCCTGTT CCGACCCTGC

15 3061 CGCTTACCGG ATACCTGTCC GCCTTCTCC CTTCGGGAAG  
CGTGGCGCTT TCTCATAGCT

3121 CACGCTGTAG GTATCTCAGT TCGGTGTAGG TCGTTCGCTC  
CAAGCTGGGC TGTGTGCACG

20 3181 AACCCCCCGT TCAGCCCGAC CGCTGCGCCT TATCCGTAA  
CTATCGTCTT GAGTCCAACC

3241 CGGTAAGACA CGACTTATCG CCACTGGCAG CAGCCACTGG  
TAACAGGATT AGCAGAGCGA

3301 GGTATGTAGG CGGTGCTACA GAGTTCTGA AGTGGTGGCC  
TAACTACGGC TACACTAGAA

25 3361 GGACAGTATT TGGTATCTGC GCTCTGCTGA AGCCAGITAC  
CTTCGGAAAA AGAGTTGGTA

WO 03/072014

PCT/US02/16877

3421 GCTCTTGATC CGGCAAACAA ACCACCGCTG GTAGCGGTGG  
TTTTTTTGTT TGCAAGCAGC

3481 AGATTACGCG CAGAAAAAAA GGATCTCAAG AAGATCCTTT  
GATCTTTTCT ACGGGGTCTG

5 3541 ACGCTCAGTG GAACGAAAAC TCACGTTAAG GGATTTGGT  
CATGAGATTA TCAAAAAGGA

3601 TCTTCACCTA GATCCTTTTA AATTAAAAAT GAAGTTTAA  
ATCAATCTAA AGTATATATG

10 3661 AGTAAACTTG GTCTGACAGT TACCAATGCT TAATCAGTGA  
GGCACCTATC TCAGCGATCT

3721 GTCTATTTCG TTCATCCATA GTTGCTGAC TCCCCGCTGT  
GTAGATAACT ACGATACGGG

3781 AGGGCTTACC ATCTGGCCCC AGTGCTGCAA TGATACCGCG  
AGACCCACGC TCACCGGCTC

15 3841 CAGATTATC AGCAATAAAC CAGCCAGCCG GAAGGGCCGA  
GCGCAGAAGT GGTCTGCAA

3901 CTTTATCCGC CTCCATCCAG TCTATTAATT GTTGCCGGA  
AGCTAGAGTA AGTAGTTCGC

20 3961 CAGTTAATAG TTTGCGCAAC GTTGTTGCCA TTGCTACAGG  
CATCGTGGTG TCACGCTCGT

4021 CGTTTGGTAT GGCTTCATTC AGCTCCGGTT CCCAACGATC  
AAGGCGAGTT ACATGATCCC

4081 CCATGTTGTG CAAAAAAGCG GTTAGCTCCT TCGGTCTCC  
GATCGTTGTC AGAAGTAAGT

25 4141 TGGCCGAGT GTTATCACTC ATGGTTATGG CAGCACTGCA  
TAATTCCTT ACTGTCATGC

WO 03/072014

PCT/US02/16877

- 4201 CATCCGTAAG ATGCTTTTCT GTGACTGGTG AGTACTCAAC  
CAAGTCATTG TGAGAATAGT
- 4261 GTATGCGGCG ACCGAGTTGC TCTTGCCCGG CGTCAATACG  
GGATAATACC GCGCCACATA
- 5 4321 GCAGAACTTT AAAAGTGCTC ATCATTGGAA AACGTTCTTC  
GGGGCGAAAA CTCTCAAGGA
- 4381 TCTTACCGCT GTTGAGATCC AGTTCGATGT AACCCACTCG  
TGCACCCAAC TGATCTTCAG
- 4441 CATCTTTTAC TTTCACCAGC GTTCTGGGT GAGCAAAAAAC  
10 AGGAAGGCAA AATGCCGCAA
- 4501 AAAAGGGAAT AAGGGCGACA CGGAAATGTT GAATACTCAT  
ACTCTTCCTT TTTCAATATT
- 4561 ATTGAAGCAT TTATCAGGGT TATTGTCTCA TGAGCGGATA  
CATATTGAA TGTATTTAGA
- 15 4621 AAAATAAACA AATAGGGGTT CCGCGCACAT TTCCCGGAAA  
AGTGCCACCT GACGTCTAAG
- 4681 AAACCATTAT TATCATGACA TTAACCTATA AAAATAGGCG  
TATCACGAGG CCCTTTCGT

- 20 The segment rhaR through PriA was taken from the E. coli chromosome using PCR  
added HindIII and modified aligned Shine-Delgarno (SD) sequence with SalI followed by  
XbaI, a stem-loop transcriptional stop sequence, and KpnI. The PCR product was cloned  
into pUC18 using HindIII and KpnI.

25

SEQ ID NO.: 154

pMPX-71 arabinose-inducible expression vector

1 TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT  
5 GCAGCTCCCG GAGACGGTCA

61 CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCC  
TCAGGGCGCG TCAGCGGGTG

121 TTGGCGGGTG TCGGGGCTGG CTTAACTATG CGGCATCAGA  
GCAGATTGTA CTGAGAGTGC

10           181   ACCATATGCG GTGTGAAATA CCGCACAGAT GCGTAAGGAG  
            AAAATACCGC ATCAGGCGCC

241 ATTCGCCATT CAGGCTGCGC AACTGTTGGG AAGGGCGATC  
GGTGCGGGCC TCTTCGCTAT

301 TACGCCAGCT GGC GAAAGGG GGATGTGCTG CAAGGCGATT  
15 AAGTTGGGTA ACGCCAGGGT

HindIII

361 TTTCCCAGTC ACGACGTTGT AAAACGACGG CCAGTGCCAA  
GCTTCAAGCC GTCAATTGTC

20

### Stop araC

421 TGATTCGTTA CCAATTATGA CAACTTGACG GCTACATCAT  
TCACTTTTTC TTCACAACCG

481 GCACGGA ACT CGCTCGGGCT GGCCCCGGTG CATTTTAA  
25 ATACCCGCGA GAAATAGAGT

WO 03/072014

PCT/US02/16877

541 TGATCGTCAA AACCAACATT GCGACCGACG GTGGCGATAG  
GCATCCGGGT GGTGCTCAAA

601 AGCAGCTTCG CCTGGCTGAT ACGTTGGTCC TCGCGCCAGC  
TTAAGACGCT AATCCCTAAC

5 661 TGCTGGCGGA AAAGATGTGA CAGACGCGAC GCGGACAAGC  
AAACATGCTG TGCGACGCTG

721 GCGATATCAA AATTGCTGTC TGCCAGGTGA TCGCTGATGT  
ACTGACAAGC CTCGCGTACC

781 CGATTATCCA TCGGTGGATG GAGCGACTCG TTAATCGCTT  
10 CCATGCGCCG CAGTAACAAT

841 TGCTCAAGCA GATTATCGC CAGCAGCTCC GAATAGCGCC  
CTTCCCTTG CCCGGCGTTA

901 ATGATTTGCC CAAACAGGTC GCTGAAATGC GGCTGGTGCG  
CTTCATCCGG GCGAAAGAAC

15 961 CCCGATTGG CAAATATTGA CGGCCAGTGA AGCCATTAT  
GCCAGTAGGC GCGCGGACGA

1021 AAGTAAACCC ACTGGTGATA CCATTGCGGA GCCTCCGGAT  
GACGACCGTA GTGATGAATC

1081 TCTCCTGGCG GGAACAGCAA AATATCACCC GGTCGGCAAA  
20 CAAATTCG TCCCTGATTT

1141 TTCACACCC CCTGACCGCG AATGGTGAGA TTGAGAATAT  
AACCTTCAT TCCAGCGGT

1201 CGGTCGATAA AAAAATCGAG ATAACCGTTG GCCTCAATCG  
GCGTTAAACC CGCCACCAGA

25 1261 TGGGCATTAA ACGAGTATCC CGGCAGCAGG GGATCATTTT  
GCGCTTCAGC CATACTTTTC

PCT/US02/16877

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10

15

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KpnI

25

70/268

WO 03/072014

PCT/US02/16877

1801 AAGTGTAAG CCTGGGTGC CTAATGAGTG AGCTAACTCA  
CATTAAATTGC GTTGCCTCA

1861 CTGCCCCGTT TCCAGTCGGG AAACCTGTCG TGCCAGCTGC  
ATTAATGAAT CGGCCAACGC

5 1921 GCGGGGAGAG GCGGTTGCG TATTGGGCGC TCTTCGCTT  
CCTCGCTCAC TGA CTGCTG

1981 CGCTCGGTGCTC TTCGGCTGCG GCGAGCGGTA TCAGCTCACT  
CAAAGGCGGT AATACGGTTA

2041 TCCACAGAAT CAGGGGATAA CGCAGGAAAG AACATGTGAG  
10 CAAAAGGCCA GCAAAAGGCC

2101 AGGAACCGTA AAAAGGCCGC GTTGCTGGCG TTTTTCATA  
GGCTCCGCCC CCCTGACGAG

2161 CATCACAAA ATCGACGCTC AAGTCAGAGG TGGCGAAACC  
CGACAGGACT ATAAAGATAC

15 2221 CAGGCGTTTC CCCCTGGAAG CTCCTCGTG CGCTCTCTG  
TTCCGACCTT GCCGCTTACC

2281 GGATACCTGT CCGCCTTCT CCTTCGGGA AGCGTGGCGC  
TTTCTCATAG CTCACGCTGT

2341 AGGTATCTCA GTTCGGTGTA GGTCGTTCGC TCCAAGCTGG  
20 GCTGTGTGCA CGAACCCCC

2401 GTTCAGCCCG ACCGCTGCGC CTATCCGGT AACTATCGTC  
TTGAGTCAA CCCGTAAGA

2461 CACGACTTAT CGCCACTGGC AGCAGCCACT GGTAACAGGA  
TTAGCAGAGC GAGGTATGTA

25 2521 GCGGGTGCTA CAGAGTTCTT GAAGTGGTGG CCTAACTACG  
GCTACACTAG AAGGACAGTA

WO 03/072014

PCT/US02/16877

2581 TTTGGTATCT GCGCTCTGCT GAAGCCAGTT ACCTTCGGAA  
AAAGAGTTGG TAGCTCTTGA

2641 TCCGGCAAAC AAACCACCGC TGGTAGCGGT GGTTTTTTTG  
TTTGCAAGCA GCAGATTACG

5 2701 CGCAGAAAAA AAGGATCTCA AGAAGATCCT TTGATCTTTT  
CTACGGGGTC TGACGCTCAG

2761 TGGAACGAAA ACTCACGTTA AGGGATTTTG GTCATGAGAT  
TATCAAAAAG GATCTTCACC

2821 TAGATCCTTT TAAATTAAAA ATGAAGTTTT AAATCAATCT  
10 AAAGTATATA TGAGTAAACT

2881 TGGTCTGACA GTTACCAATG CTTAATCAGT GAGGCACCTA  
TCTACGCGAT CTGTCTATTT

2941 CGTTTCATCCA TAGTTGCCTG ACTCCCCGTC GTGTAGATAA  
CTACGATACG GGAGGGCTTA

15 3001 CCATCTGGCC CCAGTGCTGC AATGATACCG CGAGACCCAC  
GCTCACCGGC TCCAGATTTA

3061 TCAGCAATAA ACCAGCCAGC CGGAAGGGCC GAGCGCAGAA  
GTGGTCCTGC AACTTTATCC

3121 GCCTCCATCC AGTCTATTAA TTGTTGCCGG GAAGCTAGAG  
20 TAAGTAGTTC GCCAGTTAAT

3181 AGTTTGC GCA ACGTTGTGC CATTGCTACA GGCATCGTGG  
TGTCACGCTC GTCGTTTGGT

3241 ATGGCTTCAT TCAGCTCCGG TTCCCAACGA TCAAGGCGAG  
TTACATGATC CCCCATGTTG

25 3301 TGCAAAAAAG CGGTTAGCTC CTTCGGTCTT CCGATCGTTG  
TCAGAAGTAA GTTGGCCGCA

WO 03/072014

PCT/US02/16877

3361 GTGTTATCAC TCATGGTTAT GGCAGCACTG CATAATTCTC  
TTACTGTCAT GCCATCCGTA

3421 AGATGCTTTT CTGTGACTGG TGAGTACTCA ACCAAGTCAT  
TCTGAGAATA GTGTATGCGG

5 3481 CGACCGAGTT GCTCTTGCCC GGCCTCAATA CGGGATAATA  
CCGCGCCACA TAGCAGAACT

3541 TTAAAAGTGC TCATCATTTGG AAAACGTTCT TCGGGGCGAA  
AACTCTCAAG GATCTTACCG

3601 CTGTTGAGAT CCAGTTCGAT GTAACCCACT CGTGCACCCA  
10 ACTGATCTTC AGCATCTTTT

3661 ACTTTCACCA GCGTTTCTGG GTGAGCAAAA ACAGGAAGGC  
AAAATGCCGC AAAAAAGGGA

3721 ATAAGGGCGA CACGGAAATG TTGAATACTC ATACTCTTCC  
TTTTTCAATA TTATTGAAGC

15 3781 ATTTATCAGG GTTATTGTCT CATGAGCGGA TACATATTGG  
AATGTATTTA GAAAAATAAA

3841 CAAATAGGGG TTCCGCGCAC ATTTCCCCGA AAAGTGCCAC  
CTGACGTCTA AGAAACCATT

3901 ATTATCATGA CATTAACCTA TAAAAATAGG CGTATCACGA  
20 GGCCCTTTCG TC

The segment *araC* through *Para* was taken from pBAD24 using PCR added HindIII and modified aligned Shine-Delgarno (SD) sequence with PstI followed by SalI, XbaI, a stem-loop transcriptional stop sequence, and KpnI. The PCR product was cloned into pUC18  
25 using HindIII and KpnI.

WO 03/072014

PCT/US02/16877

SEQ ID NO.: 155

pMPX-68 melibiose-inducible expression vector

5

1 TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT  
GCAGTCCCCG GAGACGGTCA

61 CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCC  
TCAGGGCGCG TCAGCGGGTG

10 121 TTGGCGGGTG TCGGGGCTGG CTTAACTATG CGGCATCAGA  
GCAGATTGTA CTGAGAGTGC

181 ACCATATGCG GTGTGAAATA CCGCACAGAT GCGTAAGGAG  
AAAATACCGC ATCAGGCGCC

241 ATTCGCCATT CAGGCTGCGC AACTGTTGGG AAGGGCGATC  
15 GGTGCGGGCC TCTTCGTAT

301 TACGCCAGCT GCGGAAAGGG GGATGTGCTG CAAGGCGATT  
AAGTTGGGTA ACGCCAGGGT

HindIII

20 361 TTTCCCAGTC ACGACGTTGT AAAACGACGG CCAGTGCCAA  
GCTTTAGCC GGGAAACGTC

Stop MelR

421 TGGCGGCGCT GTTGCTAAG TTTGCGGTAT TGTGCGGCG  
25 ACATGCCGAC ATATTTGCCG

WO 03/072014

PCT/US02/16877

481 AACGTGCTGT AAAAACGACT ACTTGAACGA AAGCCTGCCG  
TCAGGCAAT ATCGAGAATA

541 CTTTATCGG TATCGCTCAG TAACGCGCGA ACGTGGTTGA  
TGCGCATCGC GGTAAATGTAC

5 601 TGTTTCATCG TCAATTGCAT GACCCGCTGG AATATCCCCA  
TTGCATAGTT GCGTTAAGT

661 TTGACGTGCT CAGCCACATC GTTGATGGTC AGCGCCTGAT  
CATAGTTTTC GGCAATAAAG

721 CCCAGCATCT GGCTAACATA AAATTGCGCA TGGC GCGAGA  
10 CGCTGTTTTT GTGTGTGCGC

781 GAGGTTTTAT TGACCAGAAT CGGTTCCCAG CCAGAGAGGC  
TAAATCGCTT GAGCATCAGG

841 CCAATTTTCAT CAATGGCGAG CTGGCGAATT TGCTCGTTTCG  
GACTGTTAA TTCCTGCTGC

15 901 CAGCGGCGCA CTCAAACGG GCTAAGITGC TGTGTGGCCA  
GTGATTGAT CACCATGCCG

961 TGAGTGACGT GGTTAATCAG GTCTTTATCC AGCGGCCAGG  
AGAGAAACAG ATGCATCGGC

1021 AGATTAAAAA TCGCCATGCT CTGACAGGTT CCGGTATCTG  
20 TTAGTTGGTG CGGTGTACAG

1081 GCCCAGAACA GCGTGATATG ACCCTGATTG ATATTCATT  
TTTCATTGTT GATCAGGIAT

1141 TCCACATCGC CATCGAAAGG CACATTCATT TCGACCTGAC  
CATGCCAGTG GCTGGTGGGC

1201 ATGATATGCG GTGCGCGAAA CTCAATCTCC ATCCGCTGGT  
25 ATTCCGAATA CAGCGACAGC

WO 03/072014

PCT/US02/16877

+1

MeIR

1261 GGGCTGCGGG TCTGTTTTTC GTCGCTGCTG CACATAAACG  
5 TATCTGTATT CATGGATGGC

1321 TCTCTTTCCT GGAATATCAG AATTATGGCA GGAGTGAGGG  
AGGATGACTG CGAGTGGGAG  
10 1381 CACGGTTTTTC ACCCTCTTCC CAGAGGGGCG AGGGGACTCT  
CCGAGTATCA TGAGGCCGAA

1441 AACTCTGCTT TTCAGGTAAT TTATTCCCAT AAACCTCAGAT  
TTACTGCTGC TTCACGCAGG

1501 ATCTGAGTTT ATGGGAATGC TCAACCTGGA AGCCGGAGGT  
15 TTTCTGCAGA TTCGCCTGCC

SD Sall XbaI

1561 ATGATGAAGT TATTCAAGCA AGCCAGGAGG TCGTCGACTC  
TAGAGGATCC CCGCGCCCTC  
20

Stem-loop KpnI

1621 ATCCGAAAGG GCGTATTGGT ACCGAGCTCG AATTCGTAAT  
CATGGTCATA GCTGTTTCCT  
25

WO 03/072014

PCT/US02/16877

1681 GTGTGAAATT GTTATCCGCT CACAATTCCA CACAACATAC  
GAGCCGGAAG CATAAAGTGT

1741 AAAGCCTGGG GTGCCTAATG AGTGAGCTAA CTCACATTAA  
TTGCGTTGCG CTCACTGCCC

5 1801 GCTTTCCAGT CGGGAAACCT GTCGTGCCAG CTGCATTAAT  
GAATCGGCCA ACGCGCGGGG

1861 AGAGGCGGTT TCGTATTGG GCGCTCTTCC GCTTCCTCGC  
TCACTGACTC GCTGCGCTCG

10 1921 GTCGTTCCGC TCGGGCGAGC GGTATCAGCT CACTCAAAGG  
CGGTAATACG GTTATCCACA

1981 GAATCAGGGG ATAACGCAGG AAAGAACATG TGAGCAAAAG  
GCCAGCAAAA GGCCAGGAAC

2041 CGTAAAAAGG CCGCGTTGCT GCGGTTTTTC CATAGGCTCC  
GCCCCCTGA CGAGCATCAC

15 2101 AAAAATCGAC GCTCAAGTCA GAGGTGGCGA AACCCGACAG  
GACTATAAAG ATACCAGGCG

2161 TTTCCCCCTG GAAGCTCCCT CGTGCGCTCT CCTGTTCCGA  
CCCTGCCGCT TACCGGATAC

20 2221 CTGTCCGCT TTCTCCCTTC GGGAAGCGTG GCGCTTTCTC  
ATAGCTCACG CTGTAGGTAT

2281 CTCAGTTCGG TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG  
TGCACGAACC-CCCGTTACG

2341 CCCGACCGCT GCGCCTTATC CGGTAACATAT CGTCTTGAGT  
CCAACCCGGT AAGACACGAC

25 2401 TTATCGCCAC TGGCAGCAGC CACTGGTAAC AGGATTAGCA  
GAGCGAGGTA TGTAGGCGGT

WO 03/072014

PCT/US02/16877

2461 GCTACAGAGT TCTTGAAGTG GTGGCCTAAC TACGGCTACA  
CTAGAAGGAC AGTATTGGT

2521 ATCTGCGCTC TGCTGAAGCC AGTTACCTTC GGAAAAAGAG  
TTGGTAGCTC TTGATCCGGC

5 2581 AAACAAACCA CCGCTGGTAG CGGTGGTTTT TTTGTTTGCA  
AGCAGCAGAT TACGCGCAGA

2641 AAAAAAGGAT CTCAAGAAGA TCCTTTGATC TTTTCTACGG  
GGTCTGACGC TCAGTGGAAC

2701 GAAAACTCAC GTTAAGGGAT TTTGGTCATG AGATTATCAA  
10 AAAGGATCTT CACCTAGATC

2761 CTTTTAAATT AAAAATGAAG TTTTAAATCA ATCTAAAGTA  
TATATGAGTA AAC TTGGTCT

2821 GACAGITACC AATGCTTAAT CAGTGAGGCA CCTATCTCAG  
CGATCTGTCT ATTCGTTCA

15 2881 TCCATAGTTG CCTGACTCCC CGTCGTGTAG ATAACTACGA  
TACGGGAGGG CTTACCATCT

2941 GGCCCCAGTG CTGCAATGAT ACCGCGAGAC CCACGCTCAC  
CGGCTCCAGA TTTATCAGCA

3001 ATAAACCAGC CAGCCGGAAG GGCCGAGCGC AGAAGTGGTC  
20 CTGCAACTTT ATCCGCCTCC

3061 ATCCAGTCTA TTAATTGTTG CCGGGAAGCT AGAGTAAGTA  
GTTCCGCCAGT TAATAGTTTG

3121 CGCAACGTTG TTGCCATTGC TACAGGCATC GTGGTGTCAC  
GCTCGTCGTT TGGTATGGCT

25 3181 TCATTAGCT CCGGTTCCCA ACGATCAAGG CGAGTACAT  
GATCCCCCAT GTTGTGCAAA

WO 03/072014

PCT/US02/16877

3241 AAAGCGGTTA GCTCCTTCGG TCCTCCGATC GTTGTGAGAA  
GTAAGTTGGC CGCAGTGTTA

3301 TCACTCATGG TTATGGCAGC ACTGCATAAT TCTCTTACTG  
TCATGCCATC CGTAAGATGC

5 3361 TTTTCTGTGA CTGGTGAGTA CTCAACCAAG TCATTCTGAG  
AATAGTGTAT GCGGCGACCG

3421 AGTTGCTCTT GCCCGGCGTC AATACGGGAT AATACCGCGC  
CACATAGCAG AACTTTAAAA

3481 GTGCTCATCA TTGAAAAACG TTCTTCGGGG CGAAAACTCT  
10 CAAGGATCTT ACCGCTGTTG

3541 AGATCCAGTT CGATGTAACC CACTCGTGCA CCCAACTGAT  
CTTCAGCATC TTTTACTTTC

3601 ACCAGCGTTT CTGGGTGAGC AAAAACAGGA AGGCAAAATG  
CCGCAAAAAA GCGAATAAGG

15 3661 GCGACACGGA AATGTTGAAT ACTCATACTC TTCCTTTTTC  
AATATTATTG AAGCATTAT

3721 CAGGGTTATT GTCTCATGAG CGGATACATA TTTGAATGTA  
TTTAGAAAAA TAAACAAATA

3781 GGGGTTCCGC GCACATTTC CCGAAAAGTG CCACCTGACG  
20 TCTAAGAAAC CATTATTATC

3841 ATGACATTAA CCTATAAAAA TAGGCGTATC ACGAGGCCCT  
TTCGTC

25 SEQ ID NO.: 166

WO 03/072014

PCT/US02/16877

## MalE (1-370) Factor Xa NTR (43-424) FLAG

SalI +1 MalE (1-370)

1  
5 GTCGACATGAAAATAAAAAACAGGTGCACGCATCCTCGCATTATCCGCATTAACGA  
CGATGATGTTT

1 M K I K T G A R I L A L S A L T T M M F

61  
10 TCCGCCTCGGCTCTCGCCAAAATCGAAGAAGGTAAACTGGTAATCTGGATTAACG  
CGCAT

21 S A S A L A K I E E G K L V I W I N G D

121  
15 AAAGGCTATAACGGTCTCGCTGAAGTCGGTAAGAAATTCGAGAAAGATACCGGAA  
TTAAA

41 K G Y N G L A E V G K K F E K D T G I K

181  
20 GTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATTCACAGGTTGCGGCAA  
CTGGC

61 V T V E H P D K L E E K F P Q V A A T G

WO 03/072014

PCT/US02/16877

241

GATGGCCCTGACATTATCTTCTGGGCACACGACCGCTTTGGTGGCTACGCTCAATC  
TGGC

81 D G P D I I F W A H D R F G G Y A Q S G

5

301

CTGTTGGCTGAAATCACCCGGA CAAAGCGTTCCAGGACAAGCTGTATCCGTTTA  
CCTGG

101 L L A E I T P D K A F Q D K L Y P F T W

10

361

GATGCCGTACGTTACAACGGCAAGCTGATTGCTTACCCGATCGCTGTTGAAGCGTT  
ATCG

121 D A V R Y N G K L I A Y P I A V E A L S

15

421

CTGATTTATAACAAAGATCTGCTGCCGAACCCGCCAAAAACCTGGGAAGAGATCC  
CGGCG

141 L I Y N K D L L P N P P K T W E E I P A

20

481

CTGGATAAAGAACTGAAAGCGAAAGGTAAGAGCGCGCTGATGTTCAACCTGCAAG  
AACCG

161 L D K E L K A K G K S A L M F N L Q E P

25

WO 03/072014

PCT/US02/16877

541

TACTTCACCTGGCCGCTGATTGCTGCTGACGGGGTTATGCGTTCAAGTATGAAAA  
CGGC

181 Y F T W P L I A A D G G Y A F K Y E N G

5

601

AAGTACGACATTAAAGACGTGGGCGTGGATAACGCTGGCGCAAAGCGGGTCTGA  
CCTTC

201 K Y D I K D V G V D N A G A K A G L T F

10

661

CTGGTTGACCTGATTAACAAACACATGAATGCAGACACCGATTACTCCATCG  
CAGAA

221 L V D L I K N K H M N A D T D Y S I A E

15

721

GCTGCCTTTAATAAAGGCGAAACAGCGATGACCATCAACGGCCCGTGGGCATGGT  
CCAAC

241 A A F N K G E T A M T I N G P W A W S N

20

781

ATCGACACCAGCAAAGTGAATTATGGTGTAACGGTACTGCCGACCTTCAAGGGTC  
AACCA

261 I D T S K V N Y G V T V L P T F K G Q P

25

WO 03/072014

PCT/US02/16877

841

CTCAAACCGTTCGTTGGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTCCGAACA  
AAGAG

281 S K P F V G V L S A G I N A A S P N K E

5

901

CTGGCGAAAGAGTTCCTCGAAAACTATCTGCTGACTGATGAAGGTCTGGAAGCGG  
TTAAT

301 L A K E F L E N Y L L T D E G L E A V N

10

961

AAAGACAAACCGCTGGGTGCCGTAGCGCTGAAGTCTTACGAGGAAGAGTTGGCGA  
AAGAT

321 K D K P L G A V A L K S Y E E E L A K D

15

1021

CCACGTATTGCCGCCACCATGGAACGCCAGAAAGGTGAAATCATGCCGAACA  
TCCCG

341 P R I A A T M E N A Q K G E I M P N I P

20

Factor Xa +43 NTR

1081

CAGATGTCGCTTTCTGGTATGCCGTGCTGATCGAAGCCCGCACCTCGGAATCCGA  
CACG

25

361 Q M S A F W Y A V L I E A R T S E S D T

WO 03/072014

PCT/US02/16877

1141

GCAGGGCCCAACAGCGACCTGGACGTGAACACTGACATTTATTCCAAGGTGCTGG  
TGACT

5           381     A G P N S D L D V N T D I Y S K V L V T

1201

GCTATATACCTGGCACTCTTCGTGGTGGCACTGTGGGCAACTCCGTGACAGCCTT  
CACT

10           401     A I Y L A L F V V G T V G N S V T A F T

1261

CTAGCGCGGAAGAAGTCACTGCAGAGCCTGCAGAGCACTGTGCATTACCACCTGG  
GCAGC

15           421     L A R K K S L Q S L Q S T V H Y H L G S

1321

CTGGCACTGTGCGACCTGCTTATCCTTCTGCTGGCCATGCCCGTGGAGCTATACAA  
CTTC

20           441     L A L S D L L I L L L A M P V E L Y N F

1381

ATCTGGGTACACCATCCCTGGGCCCTTTGGGGACGCTGGCTGCCGTGGCTACTATTT  
CCTG

25           461     I W V H H P W A F G D A G C R G Y Y F L

PCT/US02/16877

CGTGATGCCTGCACCTATGCCACAGCCCTCAATGTAGCCAGCCTGAGTGTGGAGC  
GCTAC

5 481 R D A C T Y A T A L N V A S L S V E R Y

TTGGCCATCTGCCATCCCTTCAAGGCCAAGACCTCATGTCCCGCAGCCGCACCA  
AGAAA

10            501      L A I C H P F K A K T L M S R S R T K K

TTCATCAGTGCCATATGGCTAGCTTCGGCGCTGCTGGCTATACCCATGCTTTTCAC  
CATG

15            521       F I S A I W L A S A L L A I P M L F T M

GGCCTGCAGAACCGCAGTGGTGACGGCACGCACCCTGGCGGCCTGGTGTGCACAC  
CCATT

20            541        G L Q N R S G D G T H P G G L V C T P I

GTGGACACAGCCACTGTCAAGGTCGTCATCCAGGTTAACACCTTCATGTCCTTCCT  
GTTT

25            561       V D T A T V K V V I Q V N T F M S F L F

WO 03/072014

PCT/US02/16877

1741

CCCATGTTGGTCATCTCCATCCTAAACACCGTGATTGCCAACAACTGACAGTCAT  
GGTG

5            581     P M L V I S I L N T V I A N K L T V M V

1801

CACCAGCCGCCGAGCAGGGCCGAGTGTGCACCGTGGGCACACACAACGGTTTAG  
AGCAC

10           601     H Q A A E Q G R V C T V G T H N G L E H

1861

AGCACGTTCAACATGACCATCGAGCCGGTCTGTCTCAGGCCCTGCGCCACGGAG  
TCCTC

15           621     S T F N M T I E P G R V Q A L R H G V L

1921

GTCTACGTGCTGTGGTCATTGCCTTTGTGGTCTGCTGGCTGCCCTACCACGTGCG  
ACGC

20           641     V L R A V V I A F V V C W L P Y H V R R

1981

CTGATGTTCTGCTATATCTCGGATGAACAGTGGACTACGTTCTCTTCGATTCTA  
CCAC

25           661     L M F C Y I S D E Q W T T F L F D F Y H

WO 03/072014

PCT/US02/16877

2041

TATTTCATACATGCTAACCAACGCTCTCTTCTACGTCAGCTCCGCCATCAATCCCAT  
CCTC

5            681     Y F Y M L T N A L F Y V S S A I N P I L

2101

TACAACCTGGTCTCCGCCAACTCCGCCAGGCTTTCTGTCCACGCTGGCCTGCCT  
TTGT

10           701     Y N L V S A N F R Q V F L S T L A C L C

2161

CCTGGGTGGCGCCACCGCCGAAAGAAGAGGCCAACGTTCTCCAGGAAGCCCAACA  
GCATG

15           721     P G W R H R R K K R P T F S R K P N S M

NotI

2221

TCCAGCAACCATGCCTTTTCCACCAGCGCCACCCGGGAGACCCTGTACgcgggccgca

20           741     S S N H A F S T S A T R E T L Y A A A

Flag           stop   KpnI

GATTATAAAGATGACGATGACAAATAATAAGGTACC

D Y K D D D K \* \*

WO 03/072014

PCT/US02/16877

SEQ ID NO.: 167

5 MalE (1-28) Factor Xa NTR (43-424) FLAG

SalI +1 MalE leader (1-28)

1  
10 gtcgacATGAAAATAAAAAACAGGTGCACGCATCCTCGCATTATCCGCATTAACGACG  
ATGATGTTT

1 M K I K T G A R I L A L S A L T T M M F

Factor Xa +43 NTR

15 61  
TCCGCCTCGGCTCTCGCCAAAATCATCGAAGCCCGCACCTCGGAATCCGACACGG  
CAGGG

21 S A S A L A K I I E A R T S E S D T A G

20 121  
CCCAACAGCGACCTGGACGTGAACACTGACATTTATTCCAAGGTGCTGGTGACTG  
CTATA

41 P N S D L D V N T D I Y S K V L V T A I

WO 03/072014

PCT/US02/16877

181

TACCTGGCACTCTTCGTGGTGGGCACTGTGGGCACTCCGTGACAGCCTTCACTCT  
AGCG

61 Y L A L F V V G T V G N S V T A F T L A

5

241

CGGAAGAAAGTCACTGCAGAGCCTGCAGAGCACTGTGCATTACCACCTGGGCAGCC  
TGGCA

81 R K K S L Q S L Q S T V H Y H L G S L A

10

301

CTGTCGGACCTGCTTATCCTTCTGCTGGCCATGCCCGTGGAGCTATACAACCTTCAT  
CTGG

101 L S D L L I L L L A M P V E L Y N F I W

15

361

GTACACCATCCCTGGGCCTTTGGGGACGCTGGCTGCCGTGGCTACTATTTCCTGCG  
TGAT

121 V H H P W A F G D A G C R G Y Y F L R D

20

421

GCCTGCACCTATGCCACAGCCCTCAATGTAGCCAGCCTGAGTGTGGAGCGCTACT  
TGCC

141 A C T Y A T A L N V A S L S V E R Y L A

25

PCT/US02/16877

ATCTGCCATCCCTTCAAGGCCAAGACCCTCATGTCCCGCAGCCGCACCAAGAAAT  
TCATC

5

AGTGCCATATGGCTAGCTTCGGCGCTGCTGGCTATACCCATGCTTTTCACCATGGG  
CCTG

10

CAGAACCGCAGTGGTGACGGCACGCACCCTGGCGGCCTGGTGTGCACACCCATTG  
TGGAC

15

ACAGCCACTGTCAAGGTCGTCATCCAGGTAAACACCTTCATGTCCTTCCTGTTTCC  
CATG

20

TTGGTCATCTCCATCCTAAACACCGTGATTGCCAACAACTGACAGTCATGGTGCA  
CCAG

25

WO 03/072014

PCT/US02/16877

781

GCCGCCGAGCAGGGCCGAGTGTGCAACCGTGGGCACACACAACGTTT  
AGAGCACA  
GCACG

261 A A E Q G R V C T V G T H N G L E H S T

5

841

TTCAACATGACCATCGAGCCGGGTCTGTCCAGGCCCTGCGCCACGGAGTCTCG  
TCTTA

281 F N M T I E P G R V Q A L R H G V L V L

10

901

CTGTGCTGTGGTCATTGCCTTTGTGGTCTGCTGGCTGCCCTACCA  
CGTGCGACGCCT  
GATG

301 R A V V I A F V V C W L P Y H V R R L M

15

961

TTCTGCTATATCTCGGATGAACAGTGGACTACGTTCTCTTCGATT  
TCTACCACTA  
TTTC

321 F C Y I S D E Q W T T F L F D F Y H Y F

20

1021

TACATGCTAACCAACGCTCTCTTCTACGTCAGCTCCGCCATCAAT  
CCCATCCTCTA  
CAAC

341 Y M L T N A L F Y V S S A I N P I L Y N

25

WO 03/072014

PCT/US02/16877

1081

CTGGTCTCCGCCAACTTCCGCCAGGTCTTTCTGTCCACGCTGGCCTGCCTTTGTCC  
TGGG

361 L V S A N F R Q V F L S T L A C L C P G

5

1141

TGGCGCCACCGCCGAAAGAAGAGGCCAACGTTCTCCAGGAAGCCCAACAGCATGT  
CCAGC

381 W R H R R K K R P T F S R K P N S M S S

10

Notl Flag

1201

AACCATGCCTTTTCCACCAGCGCCACCCGGGAGACCCTGTACggggccgcaGATTATA  
AA

15

401 N H A F S T S A T R E T L Y A A A D Y K

stop KpnI

GATGACGATGACAAATAATAAGGTACC

D D D D K

20

SEQ ID NO.: 169

MalE (1-370) Factor Xa NTR (43-424) TrxA (2-109) FLAG

WO 03/072014

PCT/US02/16877

## SalI +1 MalE (1-370)

1  
GTCGACATGAAAATAAAAAACAGGTGCACGCATCCTCGCATTATCCGCATTAACGA  
5 CGATGATGTTT

1 MKIKTGARILALSALTMMF

61  
TCCGCCTCGGCTCTCGCCAAAATCGAAGAAGGTAAACTGGTAATCTGGATTAAACG  
10 GCGAT

21 SASALAKIEEGKLVIWINGD

121  
AAAGGCTATAACGGTCTCGCTGAAGTCGGTAAGAAATTCGAGAAAGATACCGGAA  
15 TTAAA

41 KGYNGLA EVGKKFEKDTGIK

181  
GTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATCCCACAGGTTGCGGCAA  
20 TGGC

61 VTVEHPDKLEEKFPQVAATG

241  
GATGGCCCTGACATTATCTTCTGGGCACACGACCGCTTTGGTGGCTACGCTCAATC  
25 TGGC

PCT/US02/16877

81 D G P D I I F W A H D R F G G Y A Q S G

301

CTGTTGGCTGAAATCACCCCGGACAAAGCGTTCCAGGACAAGCTGTATCCGTTA  
5 CCTGG

101 L L A E I T P D K A F Q D K L Y P F T W

361

10 GATGCCGTACGTTACAACGGCAAGCTGATTGCTTACCCGATCGCTGTTGAAGCGTT  
ATCG

121 DAVRYNGKLIAYPIAVEALS

421

CTGATTTATAACAAAGATCTGCTGCCGAACCCGCCAAAAACCTGGGAAGAGATCC  
15 CGGCG

141 L I Y N K D L L P N P P K T W E E I P A

481

CTGGATAAAGAACTGAAAGCGAAAGGTAAGAGCGCGCTGATGTTCAACCTGCAAG  
20 AACCG

161 L D K E L K A K G K S A L M F N L Q E P

541

25 TACTTCACCTGGCCGCTGATTGCTGCTGACGGGGGTTATGCGTTCAAGTATGAAAA  
CGGC

WO 03/072014

PCT/US02/16877

181     Y F T W P L I A A D G G Y A F K Y E N G

601

AAGTACGACATTAAAGACGTGGGCGTGGATAACGCTGGCGCGAAAGCGGGTCTGA  
5     CCTTC

201     K Y D I K D V G V D N A G A K A G L T F

661

CTGGTTGACCTGATTA AAAACAAACACATGAATGCAGACACCGATTACTCCATCG  
10     CAGAA

221     L V D L I K N K H M N A D T D Y S I A E

721

GCTGCCTTTAATAAAGGCGAAACAGCGATGACCATCAACGGCCCGTGGGCATGGT  
15     CCAAC

241     A A F N K G E T A M T I N G P W A W S N

781

ATCGACACCAGCAAAGTGAATTATGGTGTAAACGGTACTGCCGACCTTCAAGGGTC  
20     AACCA

261     I D T S K V N Y G V T V L P T F K G Q P

841

TCCAAACCGTTCGTTGGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTCCGAACA  
25     AAGAG

PCT/US02/16877

901

5 TTAAT

961

10 AAGAT

1021

15 TCCCG

Factor Xa +43 NTR

1081

20 CAGATGTCCGCTTTCTGGTATGCCGTGCTGATCGAAGCCCGCACCTCGGAATCCGA  
CACG

361 Q M S A F W Y A V L I E A R T S E S D T

WO 03/072014

PCT/US02/16877

1141

GCAGGGCCCAACAGCGACCTGGACGTGAACACTGACATTTATTCCAAGGTGCTGG  
TGA CT

381    A G P N S D L D V N T D I Y S K V L V T

5

1201

GCATATACCTGGCACTCTTCGTGGTGGGCACTGTGGCAACTCCGTGACAGCCTT  
CA CT

401    A I Y L A L F V V G T V G N S V T A F T

10

1261

CTAGCGCGGAAGAAGTCACTGCAGAGCCTGCAGAGCACTGTGCATTACCACCTGG  
GCAG C

421    L A R K K S L Q S L Q S T V H Y H L G S

15

1321

CTGGCACTGTCGGACCTGCTTATCCTTCTGCTGGCCATGCCCGTGGAGCTATACAA  
CTTC

441    L A L S D L L I L L L A M P V E L Y N F

20

1381

ATCTGGGTACACCATCCCTGGGCCTTTGGGGACGCTGGCTGCCGTGGCTACTATT  
CCTG

461    I W V H H P W A F G D A G C R G Y Y F L

25